Universidade de Aveiro Departamento de Biologia
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Llarena Reino

Controlo sanitário de parasitas de peixes nas pescarias do Atlântico

Sanitary control of fish muscle parasites in Atlantic fisheries

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutora (Programa Doutoral em Biologia; Ramo Biologia Marinha), realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, do Doutor José Vitor de Sousa Vingada, Professor Auxiliar do Departamento de Biologia da Universidade do Minho e Professor Auxiliar e Investigador Integrado do CESAM, e do Doutor Santiago Pascual del Hierro, Cientista Titular do Grupo de Ecología y Biodiversidad Marina (ECOBIOMAR) do Instituto de Investigaciones Marinas (Consejo Superior de Investigaciones Científicas, CSIC), Espanha.

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Para Pol y Josep,
quienes no han dejado
de inspirarme y motivarme,
y a quienes debo,
entre otras muchas cosas,
gran parte del tiempo dedicado
a estas páginas

o júri

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palavras-chave *Anisakis*, pescado, parasita, indústria, segurança alimentar, saúde pública

resumo

A indústria pesqueira Europeia é uma das principais atividades económicas do mundo. Os parasitas marinhos com relevância em termos de saúde pública e ao nível da indústria constituem uma questão crucial nos principais mercados Europeus, devido a três razões principais (1) a presença de um número crescente de perturbações alérgicas e gastrointestinais causadas por infecções parasitárias de origem alimentar, (2) o impacto comercial e as perdas económicas resultantes do elevado volume de rejeições, e (3) a aplicação do Regulamento (CE) 178/2002, segundo a qual "o pescado com parasitas visíveis é impróprio para consumo humano". Durante os últimos anos, com a entrada em vigor dos regulamentos Europeus e dos Estados Membros sobre alimentos, e especificamente sobre os produtos da pesca, e uma vez que a corresponsabilidade da qualidade e da segurança dos alimentos compete à indústria, a indústria pesqueira incorporou os programas de Análise do Risco e Pontos de Controlo Críticos (HACCP) nas suas competências em relação à cadeia alimentar. Consequentemente, tudo isto permitiu alcançar progressos significativos relativos à prevenção dos parasitas nos produtos da pesca. No entanto, há uma falta de consenso e de normalização sobre o tipo de inspeção de parasitas nas companhias pesqueiras, e não existe um modus operandi preciso e eficiente que seja aceite e implementado como técnica de rotina pela indústria. O atual quadro jurídico da UE definido pelos regulamentos zoo sanitários, o parecer do painel científico da Autoridade Europeia para a Segurança dos Alimentos (AESA), bem como o pacote da Higiene Alimentar entre outros, proporcionaram uma base sobre a qual o sector das pescas centra a sua actividade. Por conseguinte, a presente dissertação foi direccionada por todas estas considerações no decurso da sua execução. Este contexto conduziu-nos a realizar uma prospecção meticulosa, inovadora e multidisciplinar, como ferramenta fundamental para uma abordagem integrativa e pró-activa de gestão de riscos, entrando em linha de conta com as principais exigências dos novos mercados em relação à indústria pesqueira, e com as carências e necessidades do sector da pesca em relação ao impacto dos parasitas mais relevantes aos níveis comercial e de saúde pública.

A avaliação técnica e numerosos testes de laboratório exaustivos dos métodos qualitativos oficiais de detecção de parasitas mais utilizados no processamento do pescado (transiluminação, inspeção visual), demonstraram baixos níveis de fiabilidade. Trabalhos de investigação desenvolvidos em paralelo permitiram desenvolvimentos científicos inovadores, melhorias tecnológicas para fins de diagnóstico e a otimização dos procedimentos de detecção vigentes. Estas melhorias foram apresentadas num formato mais acessível, de mais fácil compreensão e manuseio para a sua inclusão nos programas de auto-controlo na indústria pesqueira. Por outro lado, o amplo trabalho de inspecção realizado nas espécies de peixe comerciais mais importantes permitiu chegar a um conhecimento mais aprofundado de três grupos importantíssimos de parasitas que estão a ter um impacto considerável sobre o sector das pescas; microsporídeos, anisaquídeos e copépodes. Finalmente, o desenvolvimento e aplicação prática de duas ferramentas inovadoras para a gestão de parasitoses (um sistema de avaliação preditiva em lotes de peixe, e um modelo de transmissão de conhecimento em formato web), úteis

para as empresas pesqueiras, autoridades sanitárias e público em geral, revelaram-se bons exemplos de como se pode contribuir para estimular o intercâmbio de ideias entre as partes interessadas, como melhorar a eficácia dos sistemas de inspeção, e especialmente de como converter as descobertas científicas e os avanços tecnológicos em êxitos industriais e comerciais.

A excelência científica requer investimentos em PD&I, a fim de adquirir e expandir uma base científica sólida para a política, vigilância e regulamentação da segurança dos alimentos, e também para ajudar as indústrias a alcançar um plano de prevenção de modo a que possam oferecer produtos de maior valor acrescentado. A intensa atividade diária de exportação nacional e internacional realizada nos mais importantes portos pesqueiros de Portugal e no porto de pesca de Vigo (Galiza), requer que medidas de controlo estritas, baseadas nos avanços tecnológicos e científicos mais recentes, sejam integradas nos programas pró-activos de auto-controlo das companhias pesqueiras. Ainda assim, estas medidas devem incluir ações corretivas eficazes e ações de prevenção, perante a detecção de infecções graves nas partes comestíveis dos peixes, garantindo assim aos consumidores finais produtos com o mais alto nível de qualidade e segurança.

keywords *Anisakis*, fish, parasite, industry, food safety, public health

abstract

European fisheries represent one of the leading economic activities in the world. Marine parasites with public health and industrial concern have become a key issue in major European markets, due to three main reasons: (1) the presence of a reported increasing number of allergic and gastrointestinal disorders caused by fish-borne parasitic infections, (2) the commercial impact and high economic losses due to fish rejections, and (3) the applicability of Regulation EC 178/2002, which states that "fish with visible parasites is unfit for human consumption". Over the last few years, since the entry into force of European and Member States regulations on food and specifically fishery products, co-responsibility for food quality and safety has lain with food industry, which has introduced Hazard Analysis and Critical Control Points (HACCP) programs in all its actions concerning the food chain. Consequently, significant progresses have been achieved regarding the prevention of parasites in seafood products. However, there is a lack of consensus and standardization for parasite inspection at fishing companies, and no efficient and accurate modus operandi exists to be implemented and accepted by the industry as a routine technique. The EU legal framework defined by zoosanitary regulations, scientific opinions from the European Food Safety Authority (EFSA), as well as the European Hygiene Package among others, has provided a basis on which the fishing sector has focussed its activity. Accordingly, this dissertation has been driven by these considerations during the course of its execution. This context led us to carry out a meticulous horizon scanning under a multidisciplinary approach, as an overview tool in proactive risk management. This fundamental practice takes due account of the stringent requirements that new markets are demanding to fishing industry, and the lacks and needs of the fishing sector with regard to the impact of the most relevant parasites with public health and industrial concern.

A comprehensive technical evaluation and laboratory testing of the official parasite detection methods evidenced low reliability within the two most commonly used qualitative inspection procedures in fish processing (i.e. candling, gross visual inspection). Consistent parallel research carried out, has given as a result innovative scientific developments for diagnostic purposes and for the optimization of the current detection procedures. These technological improvements have been presented in more accessible and manageable formats for their incorporation into self-control programs at the fishing industry. Furthermore, the huge amount of inspection work carried out in the most relevant fish species, has allowed reaching a deeper knowledge concerning three very important parasite groups that are impacting on fishing industry; microsporidians, anisakids and copepods. Finally, the design and application of two innovating tools for parasite management (a scoring system for predictive assessment of fish lots, and a transfer of knowledge model presented in web format), helpful for seafood producers, policy makers and general public, are good examples of how to contribute stimulating the exchanging of ideas among stakeholders and improving the inspection scheme. They are also the best approach for helping to convert scientific findings and technological advances into industrial and commercial success.

Scientific excellence requires investment in R&D&I with regard to acquire and expand a sound scientific basis for policy and regulation on food safety, and also for helping fishing industry to achieve a

preventing plan which provides added value products. The high national and international exporting activity carried out daily from the most important fishing ports of Portugal and from the fishing Port of Vigo, requires that strict control measures based on groundbreaking scientific advances, have to be incorporated into proactive self-inspections made by seafood companies. These measures must include effective preventing and corrective actions in the edible part of heavily infected fish species, thus guaranteeing products of the highest safety and quality to final consumers.

palabras clave *Anisakis*, pescado, parásito, industria, seguridad alimentaria, salud pública

resúmen

La industria pesquera en Europa constituye una de las principales actividades económicas del mundo. Las parasitosis de origen marino con repercusiones comerciales e implicaciones en la salud pública se han convertido en un problema clave en los mercados europeos debido a tres motivos principales: (1) al incremento en el número de notificaciones de alergias y desórdenes gastrointestinales causados por infecciones parasitarias transmitidas tras el consumo de pescado, (2) al impacto comercial y las elevadas pérdidas económicas debidas a los rechazos por la presencia de parásitos visibles (y/o sus lesiones asociadas), y (3) a la aplicación del Reglamento CE 178/2002, el cual establece que "todo pescado visiblemente parasitado es considerado no apto para el consumo humano". En los últimos años, a partir de la entrada en vigor de reglamentos específicos sobre los productos de la pesca (tanto a nivel europeo como a nivel de los Estados miembros), la corresponsabilidad de la calidad y seguridad alimentaria ha recaído sobre la industria alimentaria, que consecuentemente ha incorporado programas de Análisis de Peligros y Puntos Críticos de Control (APPCC) a todas sus actuaciones entorno a la cadena alimentaria. En consecuencia, todo ello ha comportado el logro de considerables avances concernientes a la prevención de los parásitos en productos marinos. Sin embargo, la ausencia de un *modus operandi* lo suficientemente eficiente y fiable en la inspección parasitaria como para ser implementado y aceptado por el sector pesquero como técnica de rutina, es fiel reflejo de la falta de consenso y estandarización existente entre las compañías pesqueras. El marco legal de la UE definido por los reglamentos zoosanitarios, las opiniones científicas de la Agencia Europea de Seguridad Alimentaria (AESA), y por el Paquete de Higiene Alimentaria entre otros, ha sentado las bases sobre las que el sector pesquero ha fundamentado su actividad, y en consecuencia, sobre las que el desarrollo de la presente tesis doctoral ha focalizado su atención. Este mismo contexto es el que nos ha llevado a realizar un meticuloso "horizon scanning" bajo un enfoque multidisciplinario y a modo de herramienta "radar". Este instrumento resulta fundamental para la gestión proactiva de riesgos, y debe tener en cuenta las principales exigencias de los nuevos mercados de la industria pesquera, así como las carencias y necesidades del sector en relación al impacto de los parásitos con mayores implicaciones sanitarias y comerciales.

La evaluación técnica y las exhaustivas pruebas de laboratorio realizadas en este trabajo para valorar la fiabilidad de los dos métodos cualitativos de detección oficiales más utilizados durante el procesado de pescado (candling e inspección visual) evidenciaron que estos procedimientos presentan un bajo nivel de fiabilidad. Las investigaciones ejecutadas en paralelo permitieron optimizar los métodos de detección de parásitos en productos de la pesca vigentes, así como desarrollar innovaciones tecnológicas con fines diagnósticos. Algunas de éstas han sido presentadas en un formato más accesible y manejable para facilitar su incorporación en los programas de autocontrol de las industrias pesqueras. Por otra parte, el amplio trabajo de inspección realizado con las especies de pescado de mayor interés comercial, permitió llegar a un conocimiento mucho más detallado de tres importantísimos grupos de parásitos que actualmente tienen un alto impacto sobre el sector; microsporidios, anisákidos y copépodos. Finalmente, el diseño, la creación y la

aplicación práctica de dos herramientas innovadoras para la gestión de parasitosis (un sistema de evaluación predictiva en lotes de pescado, y un modelo de transferencia de conocimiento en formato web) útiles para las empresas pesqueras, las autoridades sanitarias, y los consumidores, han demostrado ser buenos ejemplos de cómo contribuir a estimular el intercambio de ideas entre las partes interesadas, a mejorar la eficacia del esquema de inspección, y sobre todo a convertir los hallazgos científicos y los avances tecnológicos en éxito industrial y comercial.

La excelencia científica requiere inversión en I+D+i a fin de adquirir y expandir una base científica sólida para la normalización y vigilancia de la seguridad alimentaria, además de para ayudar a la industria pesquera a conseguir un plan de prevención que permita ofrecer productos de alto valor añadido. La intensa actividad diaria de exportación nacional e internacional que tiene lugar en el puerto pesquero de Vigo y en los puertos pesqueros más importantes de Portugal, requiere que las estrictas medidas de control basadas en los avances tecnológicos y científicos más recientes sean integradas en los programas proactivos de autocontrol de las empresas pesqueras. Asimismo, estas medidas deben incluir acciones preventivas y correctivas efectivas sobre la parte comestible de los peces gravemente parasitados, garantizando así, productos con el más alto nivel de calidad y seguridad para el consumidor final.

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CHAPTER 1

General introduction

State of the art, outline and objectives

1.1. State of the art

1.1.1. Fish parasites: public health and industrial concern

European fisheries represent one of the leading economic activities in the world. Sustainable use of marine resources in seafood chains mostly requires maintenance and improvement of ecosystem health and adequate standard of living of people who depend on it, without neglecting the quality of the final product, and consumers' health and benefits.

The presence of a growing number of fish-borne parasitic infections as anisakiasis, causing gastrointestinal diseases (Nawa et al., 2005; Mineta et al., 2006) and allergic disorders in consumers (Plessis et al., 2004; Hochberg and Hamer, 2010), is influenced by the increasing tendency to consume raw, undercooked or improperly processed seafood products (Chai et al., 2005). Even some cases of occupational asthma in fish-farming workers, have been closely related to the handling of parasitized fish (Plessis et al., 2004; Nieuwenhuizen et al., 2006). Over the last few years, with the creation of the European Food Safety Authority (EFSA), the entry into force of the Regulation (EC) 178/2002 and the European Hygiene Package (2004), and the establishment of member state regulations on food chain and more specifically on fishery products, significant progress has been achieved in the consideration of parasites as potential biological hazards.

Since co-responsibility for food quality and safety has lain with food industry, visual inspection has become the official method to be included within self-control programs for detecting visible parasites before market release. Fishery products that are obviously contaminated with parasites must not be placed on the market for human consumption (European Hygiene Package, EC 853/2004, Section VIII, Chapter V, Pt. D). For this purpose, in order to minimize the risk to human health from the potential presence of parasites in these products, a range of preventive control measures to be applied by the industry and food services was introduced through Hazard Analysis and Critical Control Points (HACCP) programs. As the Codex Alimentarius suggested in 2003, each individual facility should implement a food safety management system based on HACCP principles (CAC/RCP 52-2003). Aside from the potential impact on human health in the case of anisakids, parasites directly affect fish by decreasing its commercial value (Vidacek et al., 2009), as recently was recognized by the BIOHAZ Scientific Panel on the EFSA scientific opinion on risk assessment of parasites in fishery products (EFSA, 2010). The visible presence of parasites in seafood is a strong enough factor to significantly reduce the consumption of fish products, at least in the short-term. However, due to economic and technological constraints, it is currently impracticable to detect and subsequently remove all parasites that might be present in the fillets of wild-catch and industrially processed fish. This is further underlined by the fact that no efficient and accurate *modus operandi* exists to be implemented and accepted by the industry as a routine technique for product inspection regarding parasites. Fish industry emphasizes a real need for making available practical tools to harmonize methods, to systematically

diagnose quality challenges, sanitizing food products appropriately, exploiting potential synergies, and developing effective risk management strategies to introduce safe and high-quality products on the market. In addition, to date self-control programs in the fish industry are hampered by the fact that the epidemiology of fish parasites in European markets is not well understood. To this end, a continuous monitoring, information collection and exchange, and predictive and risk-based maintenance of self-control programs and policies, are considered necessary. Advanced technological surveillance programs should include accessible documents on legislation, available techniques, educational aspects, quality parameters and scientific fields.

1.1.2 Fish parasites: legal framework and scientific development

The EU legal framework defined by zoo sanitary regulations as well as the Hygiene Package, have provided a basis on which this thesis has been supported throughout the course of its execution. Official recommendations and scientific-technical documents as standards, guidelines, or codes of practices issued by the European Commission, Codex Alimentarius, FAO/OMS, and EFSA, among others, have also been an important axis when analyzing the situation from the point of view of the industry, consumers and public health inspectors in relation to fish parasites. Moreover, several opinions communicated in scientific forums, workshops, meetings, congresses and symposiums have been collected and used largely to support some of the results contained here.

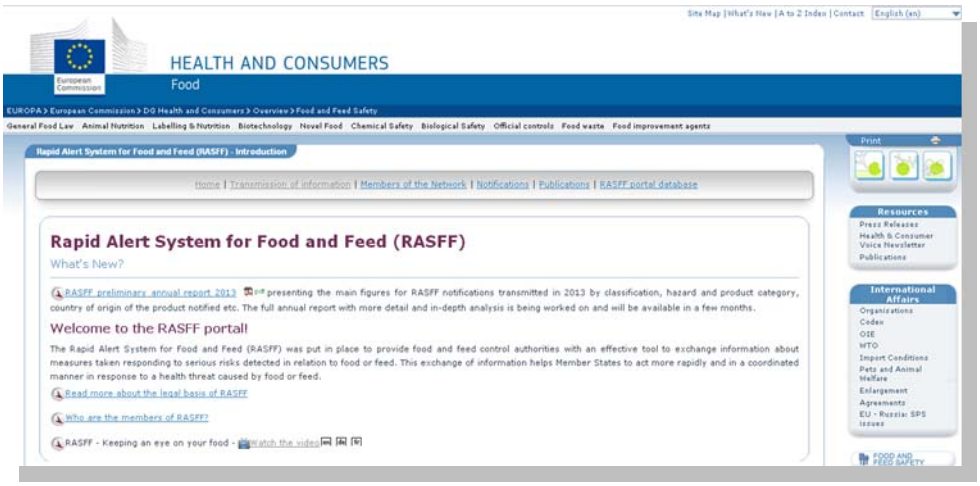
The White Paper on Food Safety (2000) reflects the key policy priority of the European Commission at assuring the highest standards of food safety in the EU. All aspects related to rapid alert systems, communication and dialogue with consumers, as well as networking with national agencies and scientific bodies, are some of the key tasks that this authority assumes. As the White Paper on Food Safety states, and as set out in Pts. 9 and 18 of Regulation (EC) 178/2002, in order to be confidence in the scientific foundation for food law, risk assessment should be undertaken in an independent, objective and transparent manner, on the basis of the available scientific information and data. With the aim of reinforcing the present system of scientific and technical support, the EFSA was established with the objective of being an independent scientific source of advice, information and risk communication, being able to be called to give opinions on contentious scientific issues, and to supply information on emerging risks with a view to their prevention (Regulation (EC) 178/2002, Pt. 33-35, 50; Regulation (EC) 853/2004, Pt. 27). Since its creation, the EFSA has been coordinating the provision of scientific advice and support for the Community's legislation and policies concerning food safety, through a Scientific Committee and Permanent Scientific Panels (e.g. Panel on Biological Hazards) formed by independent scientists (Regulation (EC) 178/2002, Pts. 45-46 and Art. 22, 28). To this end, the Authority has the task of assigning research studies necessary for the performance of its mission, using the best independent scientific resources available (Regulation (EC) 178/2002, Art. 32).

As the Codex Alimentarius recommended in 2003, the setting of critical limits for the control of hazards in fish and fishery products should be based on scientific evidence (CAC/RCP 52-2003). In this context, the establishment of microbiological criteria based on scientific risk assessment, is one of the key points that Regulation (EC) 852/2004 highlighted when laid down general rules for food business operators on the hygiene of foodstuffs. There are evidences that scientific progresses have the potential to influence on the rectification, inclusion or suppression of information promoting the updating of European law on the hygiene of foodstuffs (Commission Regulation (EC) 2074/2005, Pts. 12, 27). As an example of this, when referring to the marine parasites environment, as Council Directive 91/493/EEC of 22 July 1991 lays down in Chapter IV of Annex about parasites checks, the list of fishes subjected to freezing for a posterior cold smoking, marinated or salted process, or for a raw/almost raw consumption, only may be amended in the light of scientific data, in accordance with the procedure laid down in Art. 15 by this Directive.

Moreover, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have a strong interest in promoting national food control systems that are based upon scientific principles and guidelines, and which address all sectors of the food chain (FAO, 2003). Recent food control systems have called for science-based and transparent decision-making processes, and require access to qualified and trained personnel in disciplines such as food science and technology, chemistry, biochemistry, microbiology, veterinary science, medicine, epidemiology, agricultural sciences, quality assurance, auditing and food law. Scientific information on particular issues of concern regarding food safety is compiled by national institutions and organizations under the Scientific Co-operation (SCOOP) task (http://ec.europa.eu/food/fs/scoop/index_en.html). It involves coordination amongst Member States to provide pooled data, which are used to assist the Commission in developing EU legislation to increase protection of consumers. However, coordination of scientific information has been undertaken to build a European picture only in a limited number of areas, when in many cases it is precisely this EU dimension which is lacking to provide the information necessary for an EU risk assessment (White Paper of Food Safety, 2000, Chapter 3).

The Health and Consumers Directorate General of the European Commission manages The Rapid Alert System for Food and Feed (RASFF), which have as legal basis Regulation (EC) 178/2002. Article 50 of this Regulation has established RASFF as a network involving the Member States, the Commission as member and manager of the system, the EFSA, and also the EEA countries (Norway, Liechtenstein and Iceland). RASFF was put in place to provide food and feed control authorities with an effective tool to exchange information about measures taken when responding to serious risks detected in relation to food or feed. This exchange of information helps Member States to act more rapidly and in a coordinated manner in response to a health threat caused by food or feed. The European Commission has created the RASFF portal (http://ec.europa.eu/food/food/rapidalert/index_en.htm) to make the functioning of this system as transparent as possible to the consumer, business operators and authorities around the world. To reach this objective, RASFF considers a balance between openness and protection of information that could lead

to disproportionate economical damage. As long as dangerous products need to be recalled from the market, Member States and the European Commission immediately act to ensure products removal, and for providing the necessary information to consumers.



Taken from: http://ec.europa.eu/food/food/rapidalert/index_en.htm

Every year a new RASFF report describes its activity by classification of notifying country, hazard category (specifically including "parasitic infestation") and product category (fish and fish products, crustacean, cephalopods, bivalve molluscs, and products thereof among many others). Public awareness of the possible presence of parasites in fish products is reflected by the number of notifications under the RASFF.

Regardless of the type of manipulation prior to marketing, and the treatments applied to seafood by consumers, a determining factor in human exposure to fish parasites is their incidence in wild stocks. Consequently, identification of fishing grounds where parasites are absent or present at very low incidence is a fundamental pillar for zoonosis prevention, and one of the most important critical points within HACCP systems. This is particularly crucial in major European markets where a significant number of allergic reactions caused by zoonotic anisakids have been reported, and since many companies are offering "*Anisakis*-free" labelling in their products. Despite this, to date no protocols have been carried out to assure absence of infection. The main reason could be the difficulty for detecting and removal parasites in infected fish, especially taking into account the possibility of larvae migration from fish gut to the muscle, *intra-vitam* or subsequently to host dead. Although scientific opinion on risk assessment of parasites in fishery products (EFSA, 2010) expressed lack of knowledge on when, under what conditions and in which fish species it may occur, this fact has been mostly related to ecological and immunological factors operating in living fish, to physiological trade-off of parasites, or to biochemical *post-mortem* changes occurring in autolytic fish (Karl, 2008). The assessment and management of risks related to these food-borne hazards for ensuring a safe and high-quality seafood chain, has become a major key issue for European stakeholders. Therefore, well-planned and auspicious self-control programs which guarantee parasite-free or, at least,

effective diagnostic and management measures for parasite removal in fishing stocks and products, can provide much higher added value to the seafood chain, from net to the plate.

The implementation of the latest investigations on board fishing vessels, in fish processing plants or in the market, represents an exceptional opportunity for research institutions so they can industrially introduce and test knowledge. The promotion of effective transfer of know-how, new techniques and processes in a two-way flow, has the aim of improving seafood safety and quality standards, and ensuring the continuity of applied research work in the field of marine products and sub-products.

Recently, the European Community's Seventh Framework Programme launched a funding scheme under the Knowledge Based Bio-Economy concept (KBBE), which drives the new EU 2020 strategy (http://ec.europa.eu/research/bioeconomy/h2020/index_en.htm). Under the call FP7-KBBE-2012-6, and the action KBBE.2012.2.4-02 "Food safety and quality issues related to parasites in seafood", the project "Parasite risk assessment with integrated tools in EU fish production value chains" ("PARASITE", Grant agreement No. 312068, GA 312068), has become the first scientific project financed by the European Commission which addresses all aspects related to parasitic incidence in seafood products. From the outset, the conception of the project has had the main objective of further developing the understanding of food safety and quality aspects related to parasites of public health importance in seafood, and aims to attend to the research needs identified by EFSA regarding the risk of seafood-borne parasites. Therefore, it becomes clear that new scientific evidence and technological developments are considered necessary for the EU to progress in the risk reduction of these zoonotic diseases and the negative impacts which causes on seafood quality.

In conclusion, a proactive risk management strategy for addressing the threat of these biological hazards must include a set of actions under a multidisciplinary approach. Among them, there are some essential proceedings that we have been considered priority topics. First of all, the creation of databases on the basis of historical and bibliographic reviews from areas of interest is a fundamental starting point to describe potential scenarios. Simultaneously, knowing closely the current legal specifications, limits and recommendations regarding the subject matter hereof, places the stakeholder in a good position to properly perform further review and challenge of the effectiveness of current preventing and corrective measures. A comprehensive technical evaluation and laboratory testing of the detection methods in use and the mandatory compliance procedures in force, ideally should imply a sound and consistent parallel research, going beyond mere laboratorial diagnostic procedures. Thus, resultant innovative scientific developments should be disseminated to the fishing sector previously transformed into a more accessible and manageable format, as technological improvements, optimization of procedures or even the design of new tools. The organization of targeted events such as round tables among stakeholders, surveys in fish markets, and specific forums, constitute one of the best ways to identify, on a regular basis, the major needs and lacks of the sector. Furthermore, and as a final consideration, the transfer of knowledge and

dissemination of clearer and more practical information to seafood producers, policy makers and the general public among others, should be taken into account for completing a preventing plan for the achievement of excellence in seafood products, which should constitute the basis of the daily work within the fishing sector.

1.2 Outline

In the light of the above considerations, the present dissertation has been raised taking due account of the highlighted deficiencies and needs of the fishing sector, concerning the control of parasites in Atlantic commercial fish stocks. This thesis deals with general fish parasites, even though some chapters have been focused specifically on anisakids. This fact has been greatly influenced by the recommendations and the need for further investigation on these nematodes expressed by EFSA. The crucial role of scientific research in the progress of food legislation makes indispensable their mutual support for achieving success in terms of food safety.

This doctoral dissertation is divided in eleven chapters, including the current thesis contextualization (Chapter 1), and a final chapter, which presents the general conclusions (Chapter 11). Chapter 2 carries out an exhaustive horizon scanning on the management of emerging parasitic infections, as a proactive major strand in the field of risk evaluation, with the main purpose of exposing in detail the issue we intend to raise.

The central axis of the document is divided into two main parts: (a) Diagnostic Methods (chapters 3-5) and (b) Inspection (chapters 6-9). The first block of chapters, deals primarily with a detailed assessment of the procedures in use for detection of parasites. Evaluating the effectiveness of current diagnostic methodologies in the context of the complex scenario here exposed, is a critical point which has carefully been performed. The second underlying idea behind this section is a developed capacity for offering contrasted improvements, new tools or optimized diagnostic methods that may be integrated into self-control programs at the fishing industry to make easier and more effective the parasitic inspection of fish lots.

The second block of chapters (Inspection; chapters 6-9) firstly includes three cases of studies of different parasitic groups, which are based on showcase examples of meticulous scientific research. They do aim to give a representation of how to execute a complete analytic report starting from fish lots capable of being inspected. Different perspectives, work plans and procedures have been put in practice in the three cases, depending on the characteristics, conditions and final destination of each host species. Secondly, chapter 9 proposes a new work scheme for parasite predictive assessment in fish lots, which includes critical considerations to be incorporated into HACCP programs. Furthermore, through this innovative *modus*

operandi it is intended to establish a common language for evaluating parasite risk in fish inspections, among industry, inspectors and consumers.

Finally, Chapter 10 deals with the creation of the platform "PARCODE"; an example of transfer of knowledge about an innovative tool for parasite management in seafood products, whose visibility has been enabled in website format.

1.3 Objectives

The rigorous requirements that new markets demand has led fishing industry to make daily efforts in order to be able to offer products of the highest safety and quality. The main objective of this thesis has been to identify and give innovative solutions with high technological value to the specific needs and priorities of the fishing industry, concerning the presence of parasites in fish species of commercial interest. To reach this aim it was first necessary to execute an exhaustive work based on a meticulous inspection of fish lots, which was made possible through the kind cooperation of two of the world's most important fishing fleets; the Portuguese and the Galician fleets. Both the intensive inspection work done by carrying out a careful assessment of the current detection methods, and the study of parasitic incidence in the flesh of Atlantic commercial fish species, made possible a subsequent enhancement and optimization of the evaluated procedures as well as the proposal of new monitoring tools for industrial application. Our ultimate purpose has been to play a significant role in contributing to improve self-control programs within the inspection scheme currently used by the fishing industry, to guarantee safe and quality seafood products.

Considering the overall goal pointed above, this thesis had the following specific objectives:

- To carry out a detailed and complete horizon scanning, as a major strand in proactive risk management, in relation to the impact of the most relevant parasites with public health and industrial concern on the value chain of commercial fishery products.
- To evaluate the efficacy of the washing practice to remove *Anisakis* spp. from guts, and to analyse the statistical significance between the number of observable muscular parasites and gut parasites of commercial fish species, in order to assess the accuracy of the current European legislation.
- To assess and improving the artificial digestion protocol in use recommended by the Codex Alimentarius for anisakids detection in fish, with the main purpose of offering an optimized and safer procedure for fish factory workers.
- To determine the fluorescence emission pattern and the basis of the auto-fluorescence of *Anisakis simplex* larvae extracted from commercial fish specimens, with the intention of enhancing the UV-light

examination method on fish fillets, and proposing more efficient and affordable imaging tools for fish industry.

- To examine in further detail Atlantic anglerfish, *Lophius budegassa* and *Lophius piscatorius* specimens for the presence of muscular microsporidian parasites, with the purpose of integrating for the first time in the same parasite sample, site of infection, epidemiological data, phenotypic, genotypic, and fine structural characterizations.

- To provide a comprehensive response to the plea made in 2010 by the EFSA, requiring more epidemiological available information for potentially hazardous parasites, by studying and testing the efficiency and reliability of the press technique and visual inspection of fillets under an UV-light source, for detecting nematode larvae afecting commercially important fish species.

- To determine distribution, infection levels, morphological and genetic identification of pennellid specimens present in the Atlantic swordfish, *Xiphias gladius,* one of the most important commercial species marketed in the European Union.

- In absence of an inspection standard and a "*quantum satis"* statement for parasites, to design and test a novel and predictive tool for evaluating parasitic risk in the flesh of fish lots during inspections, with the aim of proposing an enhanced inspection scheme and a common language to the fishing sector.

- To revitalize and invigorate the seafood industry-inspectors-researchers-consumers relationships, and to provide understandable and tempting scientific and technical information and support, in order to help managing and mitigating the impact of zoonotic parasites present in fish stocks and fishery products in the European market.

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CHAPTER 2

Horizon scanning

Management of emerging parasitic infections

Llarena-Reino, M., Abollo, E., Regueira, M., Rodríguez, H. and Pascual, S. (2013). Horizon scanning for management of emerging parasitic infections in fishery products. Food Control, 49:49-58.

ABSTRACT

Public organizations operating in health and food-safety sectors are increasingly realizing the advantages of the long-term view of risk uncertainties associated to biological hazards, served-up in the short-term to anticipate the problem and its handling. Thus, the horizon scanning is becoming a major strand in proactive risk management and patient-consumer protection continuity. This approach was recently explained in the scientific opinion on risk assessment of parasites in fishery products by the European Food Safety Authority, EFSA (2010), followed by the launching of a funding scheme for a specific EU Framework Program Project under the Knowledge Based Bio-Economy concept, KBBE (FP7-KBBE-2012-6), which drives the new EU 2020 strategy. The aim of this paper is to examine horizon scanning issues in relation to public health and industrial concern on the presence of parasites in fishery products recorded in the Rapid Alert System for Food and Feed (RASFF) System. We focus on specific threats, targets, methods and challenges as a means of acquiring management goals and future objectives. The proposed horizon scanning identifies emerging ideas/technologies for an early handling of parasitized fish stocks/products for priority setting to inform strategic planning of stakeholders, policy-makers and health services. In order to accomplish this, a set of risk GIS maps illustrating the state of art about the presence of the zoonotic *Anisakis* spp. on commercial fish stocks of the last 65 years was firstly developed. Secondly, a program of 108 surveys among fish sellers of Galicia (NW Iberian Peninsula) were carried out with the main objective of getting information about hazard recognition, fish product management practices, quality self-controls and corrective and preventive measures in use. Additionally, during the "I International Symposium on strategies for management of parasitized seafood products" (Vigo, Spain), groups of researchers, technologists, official inspectors and industry technicians participated in round tables with 3 different perspectives: market-industry, inspection and academia. All stake-holders agreed that the *status quo* to manage fish parasites in the production-to-consumption food pathway is unsatisfactory. The central message proposed a stable network performance based on collaborative software to provide multi-level information for industrial management of parasite contaminants in fish products. The discussion group also proposed to invigorate collaborative translational research and professional training as key drivers to fuel technological innovations and tech transfer, which may help to minimize/eliminate the risk of parasites that have public health and economic impacts in fish products.

KEYWORDS

Horizon scanning; fishery products; parasite; public health; commercial value; inspection

2.1. INTRODUCTION

2.1.1. The horizon scanning concept

Horizon scanning is considered to be one of the most useful tools for strategic decision-making. It systematically anticipates, identifies and informs about emerging trends and issues and potential threats and risks. In addition, it helps policy-makers to take a longer-term strategic approach and developing new insights. Its strategic scans can be disseminated in the form of policy briefs, reports or scenarios, are used to improve operational vigilance or resilience, and develop robust strategies for decreasing risk exposure. Furthermore, it allows better preparedness and the incorporation of mitigation and management measures into the policy and decision-making processes. Finally, horizon scanning emphasizes the creation of networks and knowledge flows between people and organizations.

2.1.2. The fish parasite problem

Marine parasites constitute an important health and quality threat in fishery products (Sabater and Sabater, 2000). Since the middle 20th century, scientific evidences have confirmed the presence of a high and raising prevalence of a “dirty dozen” of parasites in wild stocks of fishery products of commercial interest around the world (Adams et al., 1997; Abollo et al., 2001; Køie, 1993; McClelland et al., 1985; Mladineo, 2001; Quijada et al., 2005; Rello et al., 2009; Smith and Wootten, 1979; Valero et al., 2006; Wharton et al., 1999). Reasons for these emerging fish diseases in fishery products are diverse. Primarily, outbreaks depend on the nature and life-cycle strategy of the parasites, but mostly on an uncontrolled ecosystem management and on new consumers feeding habits. Well-know examples of ecosystem-based implications for parasites are the outbreak spreading of *Giardia* and *Cryptosporidium* protozoans around shellfish harvesting areas due to fecal contamination by river and waste waters (Freire-Santos et al., 2000; Gómez-Couso et al., 2005), or protectionist policies for marine mammals followed by several fishing practices that may increase the recruitment of zoonotic, allergenic anisakid nematodes at fishing grounds (Abollo et al., 2001; McClelland et al., 1990; Rodriguez et al., 2009). Furthermore, the new wave of increasingly eating raw or undercooked fishery products has also epidemiological implications in industrialized countries. Specifically, *Giardia, Cryptosporidium*, some species of anisakids and more recently *Kudoa* have been recognized as human health hazards responsible for emergent zoonoses, that causes from gastro-allergic disorders in consumers (Audicana et al., 2002; Chen et al., 2008; Dick et al., 1991; Kawai et al., 2012; Smith and Wootten, 1978; Vidacek et al., 2009) to occupathional-asma in fish-farming workers (Nieuwenhuizen et al., 2006; Plessis et al., 2004). Besides these detrimental effects on public health, the presence of parasites in fishery products may also hamper the commercial value of products thus reducing its marketability (Arthur et al., 1982; Crowden and Boom, 1980; Brassard et al., 1982; Lom and Dyková, 1992; Williams and Jones, 1994; Kumaraguru et al., 1995; Woo, 1995). As an example, the economic losses among fish processing industries

caused by anisakid larvae in fish flesh have been estimated to reach several millions of dollars (Bonnell, 1994).

The “dirty dozen” genera that affect the quality and/or safety of fishery products comprise micro and macroparasites. Concerning microparasites (apart from waterborne *Giardia* and *Cryptosporidium*), the mixosporidians (*Kudoa* spp.) and the microsporidians (*Pleistophora* spp. and *Spraguea* spp.) are highly prevalent in the flesh of gadoid fish, mostly merluccidae and anglerfishes (Casal et al., 2012; Freeman et al., 2004; Leiro et al., 1996; Pascual and Abollo, 2008; Whipps and Diggles, 2006). Among the macroparasites, didymozoid trematodes occurring in scombrids (Pascual et al., 2006), cestodes (*Gymnorhynchus* spp., *Molicola* spp.) present in pomfret fish and swordfish, the cosmopolitan anisakid nematodes (*Anisakis* spp, *Pseudoterranova* spp., *Contracaecum* spp.) and crustaceans of *Pennella* spp. in the swordfish, represent the relevant target parasites during veterinary inspections of fresh and frozen products in the European fish industry.

The nematode *Anisakis* is a good candidate to be eligible as a sentinel model for targeting a horizon scanning for managing emerging parasites in fishery products. The reasons are:

i) It is by far the most prevalent macroparasite in fish products from major stocks around the world, with significant demographic infection values regardless of the host species and fishing area. Especially of concern is the fact that during fish inspections anisakids are usually found in high amount on the gut cavity (Vidacek et al., 2009), in a lower quantity on the belly flaps (Abollo et al., 2001), and sometimes in the flesh (Llarena-Reino et al., 2012; Smith, 1984; Valero et al., 2006; Wharton et al., 1999).

ii) In the last 20 years anisakids has been a trending topic within the scientific community, fish consumers and the industry dealing with biological risks in seafood products. These results from many social alarms in most southern European countries (León et al., 2006; Poli, 2005) linked with the trending record of available medical literature concerning the public health implications of anisakids in general, and the genus *Anisakis* in particular.

iii) Besides the repercussion they have on seafood safety, quality aspects in parasitized fish decrease its commercial value by affecting the aesthetic of products (Figure 2.1). This fact is hampering marketability of seafood products within a fair international trade and European consumer preferences that demand products with high standard quality (Pascual et al., 2010; Vidacek et al., 2009).

iv) Because the parasite recruitment is successfully adapted to the marine trophic webs, alterations in the ecosystem reflect changes in the epidemiological status of this hazard in fish stocks and products (Deardorff, 1991; Marcogliese, 2001; Pascual et al., 2007; Slifko et al., 2000; Wood et al.,

2010). This reinforces the idea of a management strategy enlarged from the net to the plates which also should include a study of viability of parasites in unprocessed marine fish waste used for feeding aquaculture fish, as juvenile wild fish on-grown in captivity.

v) The risk assessment of this hazard demands a management strategy as the base of a fair international trade for products of different origin and production methods. In most cases neither the strategy is implemented nor available tools are integrated in the industry.

In relation to the discussion paper on the guide interpretation of Regulation (EC) 853/2004, recently the European Commission considered necessary to carry out a consultation to seafood industries' operators regarding the regulation of consumer information on such legislation. The present work aimed to propose the elaboration of a detailed and complete horizon scanning of the situation resulting from the impact of the most relevant parasites on the value chain of commercial fishery products. To this end and following the mentioned example of the European Commission, authors decided to arrange a meticulous analysis and discussion by using the same "consultation" method with fisheries stakeholders. Thus a triple strategy was put in practice:

(1) As a previous step it was considered the elaboration of risk GIS maps illustrating the state of the art concerning the condition of commercial fish stocks during the last 65 years, regarding the effect of the zoonotic parasite: *Anisakis* spp. Nowadays, there is an increasing interest on the use of GIS as an innovative technology to combine epidemiology, statistics and geographic information, due to the assist it provides by facilitating decision making, processing and analysis of information on several multidisciplinary areas.

(2) Secondly, it was planned a program of surveys to fishmongers. The consultative and anonymous character of this methodology, the potential amount of information available that it offers, the "consumer representation" made by fish sellers, and the "intermediary" role played by them within the fishing guild (exerts great influence on the extractive sector and on consumers), were important enough reasons to choose this tool.

(3) Finally, it was carried out the organization of three round tables framed within an international symposium. Panel discussions had the objective of agglutinating scientists (round table 1), health inspectors (round table 2) and representative stakeholders from: fishing companies, the extractive sector, aquaculture businesses, restaurants, distributors, wholesalers and retailers of fish, etc (round table 3).

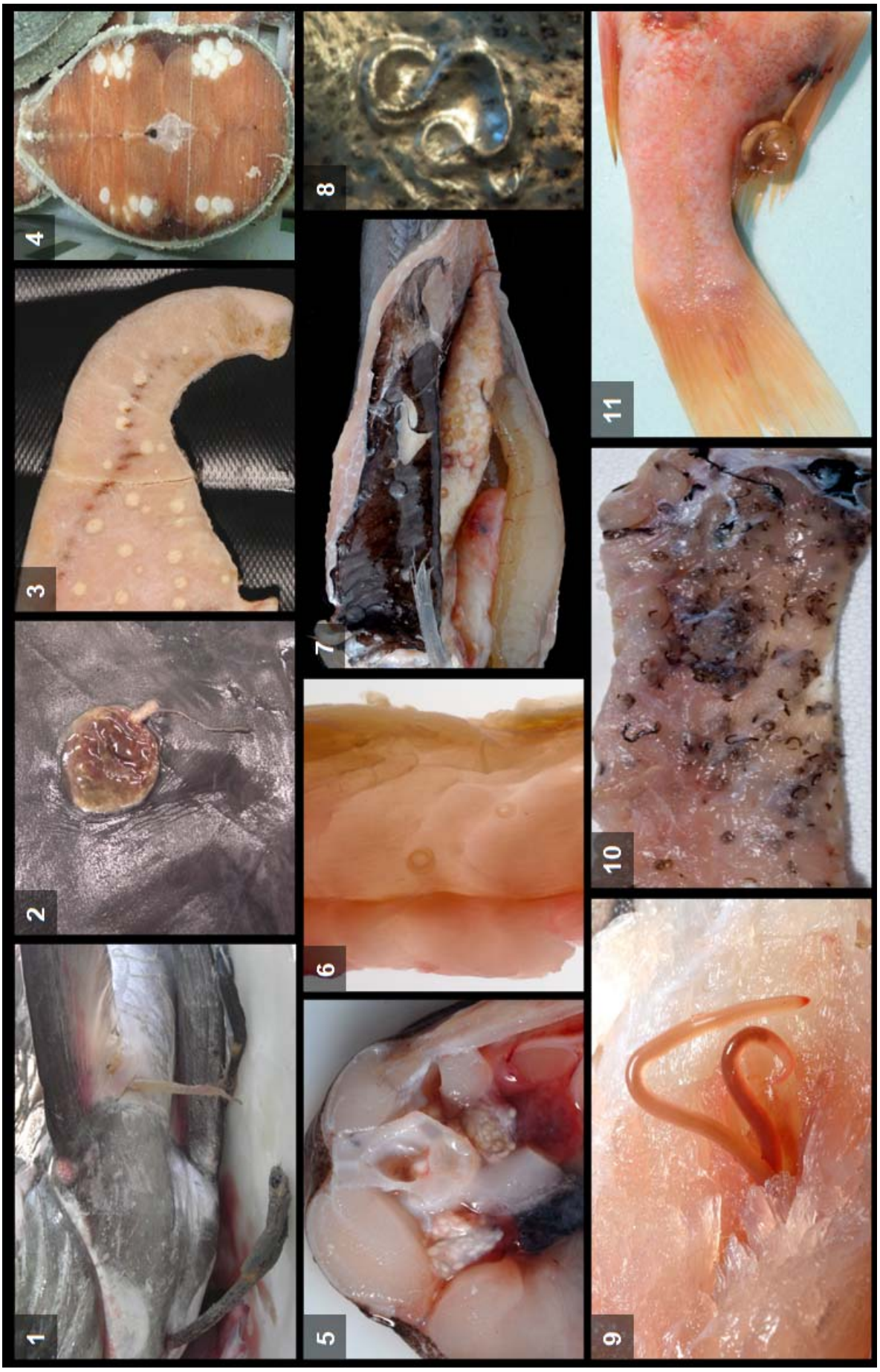
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Figure 2.1. (1-11). Macrophotographs showing unaesthetic problems associated to visible parasites found in commercial fish lots. The unaesthetic appearance that many parasites produce on seafood products represent a serious problem that has a significant impact on consumer's preferences by decreasing enormously the commercial value of affected products. Regardless of the concern for the public health, the effects that parasites causes on marketability forces seafood industry to discard large quantities of fish and to intensify quality inspection protocols on seafood products. At this point, the most valuable goals of the industry are increasing the quality of parasitized products and the consumer's confidence. **1.** Up to 3 copepods belonging to *Pennella* sp. with the anterior end anchored internally in the musculature of *Xiphias gladius*. **2.** *Pennella* sp. causing inflammatory and ulcerous wounds around the entrance hole followed by abscesses in host musculature. **3.** Large number of *Molicola* sp. within the flesh of *X. gladius*. **4.** Pseudocysts of *Kudoa* sp. in the flesh of *Salmo salar*, at times associated to *post-mortem* myoliquefaction ("milky flesh syndrome"). **5.** Microsporidian xenomas of *Spraguea lophii* infecting nervous tissues of *Lophius budegassa*, usually located along the length of the vertebral column (body), and on the medulla oblongata of the hind brain (head). **6.** Encysted larval of *Anisakis* sp. in the flesh of *Micromesistius poutassou*. **7.** Encysted larvae of *Anisakis* sp. in the gut cavity and belly flap of *M. poutassou*. **8.** Larval of *Anisakis* sp. migrating under the skin of *M. poutassou*. **9.** Larval of *Pseudoterranova decipiens* in the flesh of *Lophius piscatorius*. **10.** Old encysted (melanin capsules) larvae of *Anisakis* sp. embedded in the flesh of *Merluccius merluccius*. **11.** Copepod belonging to the the sphyriid *Sphyrion lumpi* in *Sebastes mentella*, anchored internally in the musculature surrounding fins.

The main reason why horizon scanning was used as a suitable and useful method to identify key issues of concern and provide solutions to this biological hazard, is that horizon scanning explores novel and unpredicted topics as well as persistent problems and tendencies. The practice of this technique can be undertaken by small groups of experts who are at the forefront in the area of concern with the aim of sharing their perspectives and knowledge with each other. This tool is becoming a major strand in proactive risk management and business continuity.

2.2. MATERIALS AND METHODS

EU legislation forces food market and industry to ensure, from the catch to the plate, that no contaminated fish reach the consumer. To that end stakeholders shall put in place, implement and maintain permanent procedures based on the HACCP principles (Regulations (EC) 852-854/2004; Commission Regulation (EC) 2074/2005). The European Hygiene Package (Council Directive 91/493/EEC; Commission Decision 93/140/EEC; Regulations (EC) 852-854/2004, Council Regulation (EC) 2406/96; Commission Regulation (EC) 2074/2005) and its modifications (Commission Regulations (EC) 1662-1664/2006), establishes that food business operators shall ensure that all stages of production, processing and distribution satisfy and comply with general and relevant hygiene requirements. Therefore fish industry has become responsible of the submission of fishery products for human consumption to visual inspection for the purpose of detecting visible parasites before being placed on the market. Considering the scientific literature to date and taking the European legislation in perspective, we defined the end-user prospect in a triple scheme:

2.2.1. Maps

In order to agglutinate available data illustrating the impact of parasitism by *Anisakis* spp. over fisheries, a literature search using the ISI Web of Knowledge databases was performed to compile articles published from 1947 to 2011 related to the keyword "*Anisakis*" in Atlantic Ocean. As a result a total of 929 publications were obtained and information from 104 selected papers with georeferenced samples was extracted. The resulting 1287 registers were added to a computerized database. The retrieved information covered parasite and host species, sampling size, geographic location, date, anatomical site of infection, prevalence, mean intensity, mean abundance and density of infection, and the methods used for parasite detection. According to compiled information, overall infection parameters were calculated for each FAO fishing subzone. Geographic Information Systems (GIS) software ArcGIS 9.3. was used to link epidemiological information to FAO fishing areas' vector layer. This map layer identified each fishing subzone by a unique ID polygon. A series of maps were produced to show the averages of the registered parameters of infection for each polygon in the Atlantic Area (Figure 2.2). The cartography generated included a specific set of maps showing overall demographic infection values for *Anisakis* spp. for FAO subzones and also information relative to both host orders and species of fishery importance.

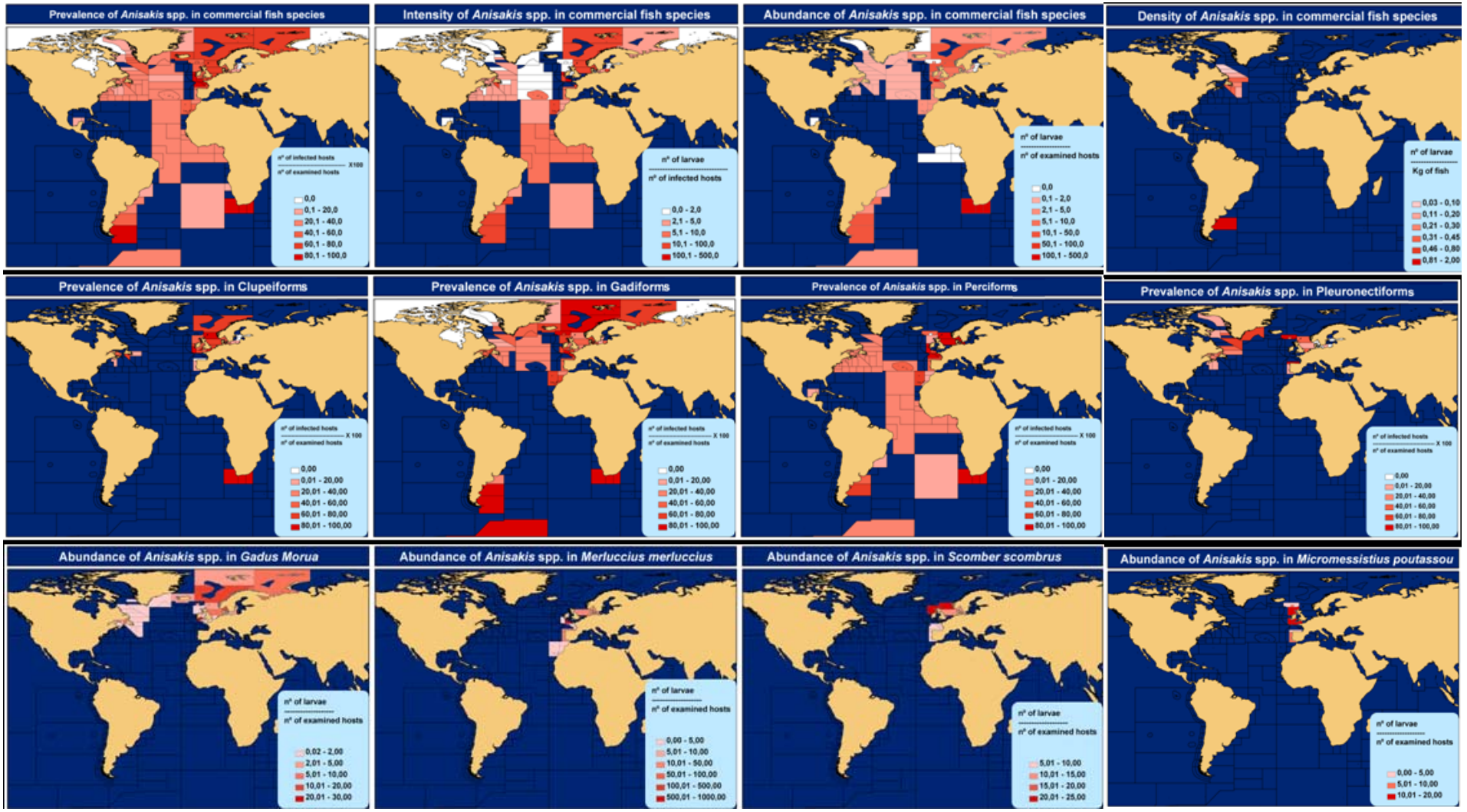

Prevalence of Anisakis spp. in commercial fish species
nº of infected hosts / nº of examined hosts X100
0,0
0,1 - 20,0
20,1 - 40,0
40,1 - 60,0
60,1 - 80,0
80,1 - 100,0
Intensity of Anisakis spp. in commercial fish species
nº of larvae / nº of infected hosts
0,0 - 2,0
2,1 - 5,0
5,1 - 10,0
10,1 - 100,0
100,1 - 500,0
Abundance of Anisakis spp. in commercial fish species
nº of larvae / nº of examined hosts
0,0
0,1 - 2,0
2,1 - 5,0
5,1 - 10,0
10,1 - 50,0
50,1 - 100,0
100,1 - 500,0
Density of Anisakis spp. in commercial fish species
nº of larvae / Kg of fish
0,03 - 0,10
0,11 - 0,20
0,21 - 0,30
0,31 - 0,45
0,46 - 0,80
0,81 - 2,00
Prevalence of Anisakis spp. in Clupeiforms
nº of infected hosts / nº of examined hosts X 100
0,00
0,01 - 20,00
20,01 - 40,00
40,01 - 60,00
60,01 - 80,00
80,01 - 100,00
Prevalence of Anisakis spp. in Gadiforms
nº of infected hosts / nº of examined hosts X 100
0,00
0,01 - 20,00
20,01 - 40,00
40,01 - 60,00
60,01 - 80,00
80,01 - 100,00
Prevalence of Anisakis spp. in Perciforms
nº of infected hosts / nº of examined hosts X 100
0,00
0,01 - 20,00
20,01 - 40,00
40,01 - 60,00
60,01 - 80,00
80,01 - 100,00
Prevalence of Anisakis spp. in Pleuronectiforms
nº of infected hosts / nº of examined hosts X 100
0,00
0,01 - 20,00
20,01 - 40,00
40,01 - 60,00
60,01 - 80,00
80,01 - 100,00
Abundance of Anisakis spp. in Gadus Morua
nº of larvae / nº of examined hosts
0,02 - 2,00
2,01 - 5,00
5,01 - 10,00
10,01 - 20,00
20,01 - 30,00
Abundance of Anisakis spp. in Merluccius merluccius
nº of larvae / nº of examined hosts
0,00 - 5,00
5,01 - 10,00
10,01 - 50,00
50,01 - 100,00
100,01 - 500,00
500,01 - 1000,00
Abundance of Anisakis spp. in Scomber scombrus
nº of larvae / nº of examined hosts
5,01 - 10,00
10,01 - 15,00
15,01 - 20,00
20,01 - 25,00
Abundance of Anisakis spp. in Micromessistius poutassou
nº of larvae / nº of examined hosts
0,00 - 5,00
5,01 - 10,00
10,01 - 20,00

Figure 2.2. Cartography that includes a specific set of maps illustrating the averages of demographic infection values for *Anisakis* spp. in each Atlantic FAO fishing subarea (1st row), and related to host orders (2nd row) and species of fishery importance (3rd row).

2.2.2. Inquiries

A program of 108 surveys to fish sellers from fish stands, whose main objective was to get information about (1) hazard recognition, (2) fish product management practices, (3) quality self-controls at points of distribution or sale, and (4) corrective/preventive measures in use, was carried out. Fish stands were placed in 17 city market squares, 20 village market squares, 4 super/hypermarkets and 4 fish shops, all located in Galicia (NW Spain). A brief description of each type of establishment aims to achieve a better understanding:

- Market square: a place where different establishments sold daily food from agriculture, livestock and fishing.

- Super/hypermarkets: self-service expansive facilities offering a wide variety of food and household products. These establishments sells fish, meat, fresh produce, dairy, and baked goods, along with shelf space reserved for canned and packaged goods as well as for various non-food items.

- Fish shop: a shop that sells fish; a fishmonger's.

The reason why there was an over-representation of market squares and an under-representation of super/hypermarkets and fish shops in the surveys is because these claimed to reflect the consumption habits of the population in the area studied. A total of 2 technicians executed the surveys as individual and anonymous interviews composed of 8 questions each one. Selected queries for interviews were previously planned and described by a group of marine scientists, parasitologists and veterinarians whose lines of research are closely linked to parasites in commercial fish species. Those questions dealt with the recognition and the presence of anisakids in fish, handling practices and with improvements in sanitary conditions of the establishments. The questions were as follows:

1. Type of establishment interviewed (city market square, village market square, super/hypermarket, and fish shop).

2. Which improvements do you consider essential to ensure sanitary and quality conditions of fish at the point of sale: hot potable water, marine water, improved cleaning, better refrigerators, rain water system with timer, better illumination, flake ice machine, refrigerated desk, individual potable water or nothing?

3. Do you eviscerate any of the following fish species or remove the hypaxial muscle before placing fish for sale? (*Engraulis encrasicholus*, *Merluccius merluccius*, *Micromesistius poutassou*, *Conger conger*, *Lophius* spp., *Lepidorhombus* spp., *Sardina pilchardus*, *Zeus faber*, *Scomber scombrus*, *Trachurus* spp., other fish species).

4. Do you eviscerate any fish species at points of sale before keeping fish overnight? (no, yes, certain species).

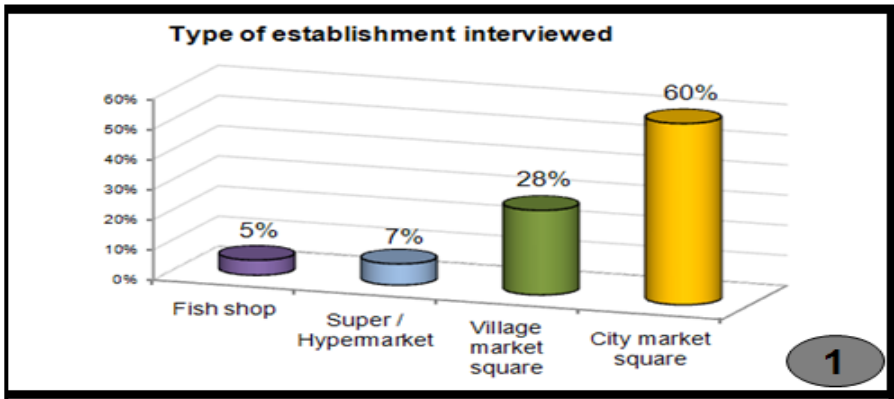
5. Do you remove the hypaxial muscle at any fish species at points of sale before keeping fish overnight? (No, yes, certain species).

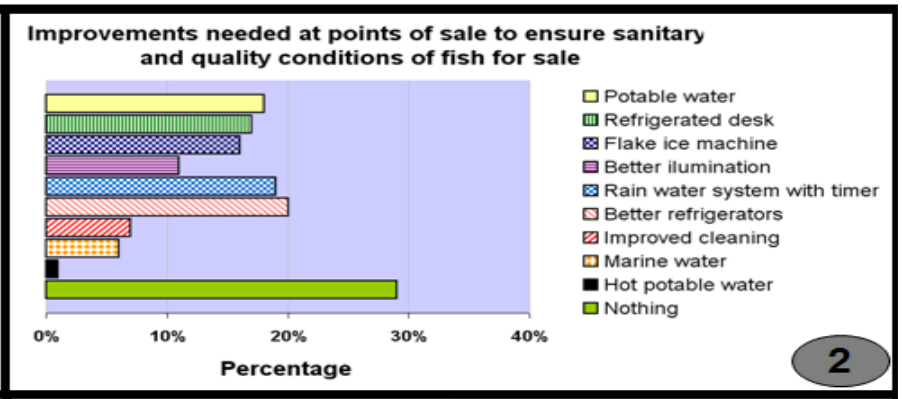
6. Do you know anisakids? (No, yes).

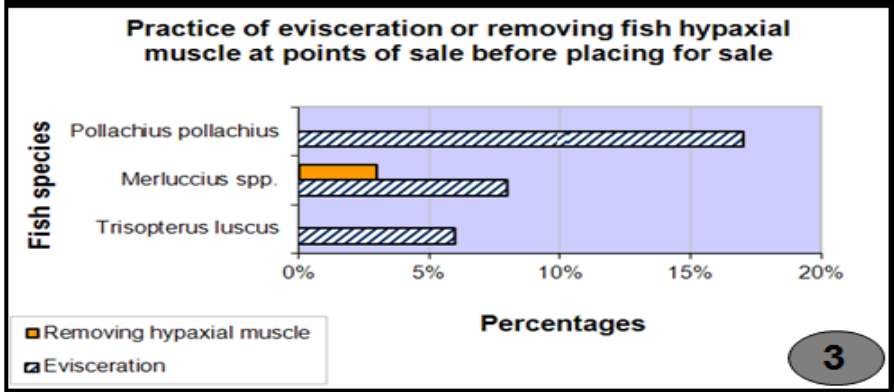
7. Do you usually reject fish species due to the presence of anisakids? (no, yes, which species?).

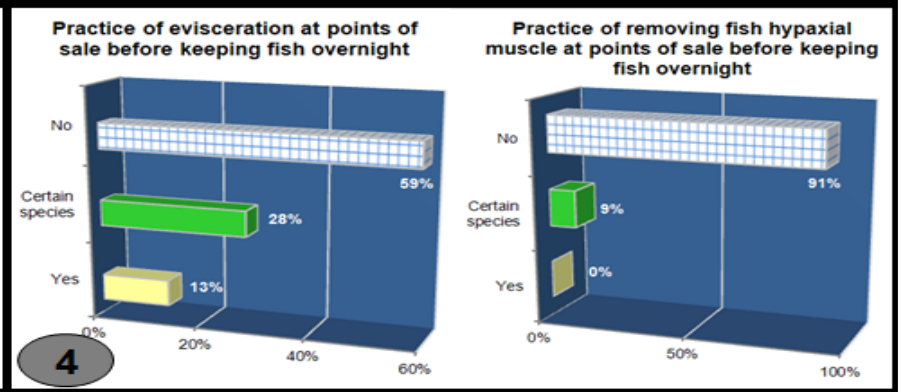
8. Do you usually have claims from consumers due to the presence of anisakids in any fish species? (no, yes, which species?).

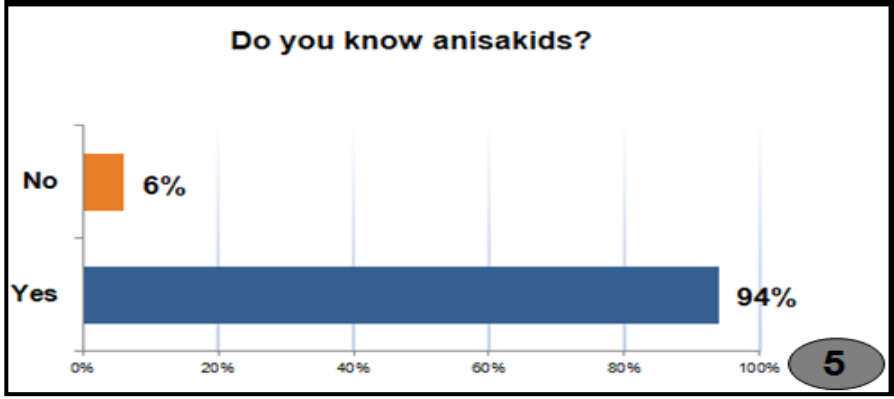
The results from the surveys performed were compiled, submitted to a descriptive analysis, worked out, compared, matched when necessary, and then represented in graphics (Figure 2.3). Furthermore, a Spearman Rank Order Correlation was carried out to test the statistical inference between sellers' rejections and consumers' claims due to fish infected by anisakids.


Type of establishment interviewed
60%
50%
40%
30%
20%
10%
0%
5%
7%
28%
60%
Fish shop
Super / Hypermarket
Village market square
City market square
1


Improvements needed at points of sale to ensure sanitary and quality conditions of fish for sale
Potable water
Refrigerated desk
Flake ice machine
Better illumination
Rain water system with timer
Better refrigerators
Improved cleaning
Marine water
Hot potable water
Nothing
0%
10%
20%
30%
40%
Percentage
2


Practice of evisceration or removing fish hypaxial muscle at points of sale before placing for sale
Fish species
Pollachius pollachius
Merluccius spp.
Trisopterus luscus
0%
5%
10%
15%
20%
Percentages
Removing hypaxial muscle
Evisceration
3


Practice of evisceration at points of sale before keeping fish overnight
No
Certain species
Yes
59%
28%
13%
0%
20%
40%
60%
Practice of removing fish hypaxial muscle at points of sale before keeping fish overnight
No
Certain species
Yes
91%
9%
0%
0%
50%
100%
4


Do you know anisakids?
No
6%
Yes
94%
0%
20%
40%
60%
80%
100%
5

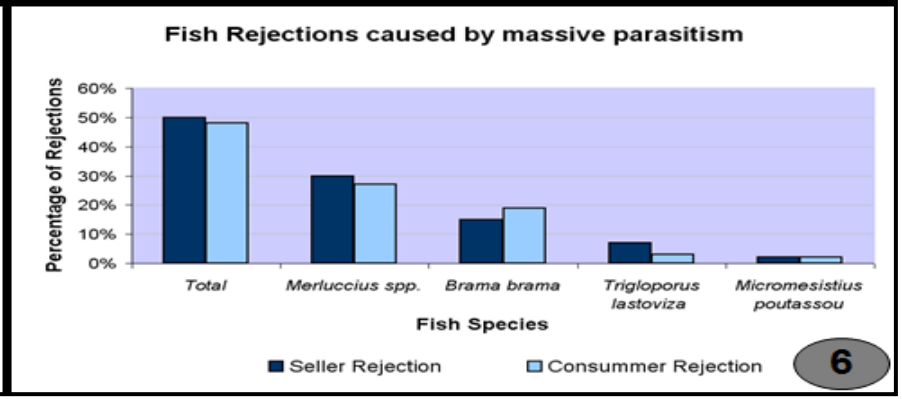

Fish Rejections caused by massive parasitism
Percentage of Rejections
60%
50%
40%
30%
20%
10%
0%
Total
Merluccius spp.
Brama brama
Trigloporus lastoviza
Micromesistius poutassou
Fish Species
Seller Rejection
Consummer Rejection
6

Figure 2.3. Graphical representation of the results obtained after carrying out a total of 108 surveys among fish sellers in Galicia, NW Spain.

2.2.3. Roundtables

The "I International Symposium on strategies for management of parasitized seafood products" gathered and organized in Vigo (Spain) in November 2010 (http://www.iim.csic.es/parcode/), had a total of 200 participants from different countries and professional areas. Among them, 30% were fisheries industry agents (from more than 50 fishing companies) including representatives of the extractive sector, aquaculture, distributors, wholesalers and retailers of fish, restaurants, etc., 30% were veterinarians responsible of inspection services for the Administration, 22% of assistants came from academic institutions, and 18% were consumers, students and independent professionals. This event have represented an important approach between scientific researchers involved in the presence of parasites in seafood, and all the agents that in any way are affected by this problem.

Parallel to the symposium, a set of round tables with 3 different groups of representative horizon scanners took place, by means of 3 different perspectives: academia, inspection and market-industry. Those 3 groups integrated (a) 12 scientific researchers, (b) 25 public health official inspectors and (c) 25 technologists from the fish industry, respectively. The round tables began with a series of individual and illustrative presentations, which included oral explanations of the current situation. In the case of scientific researchers' round table, each participant presented his point of view of the *status quo* during around 10-15 min. In the cases of official inspectors' and fish industries' round tables, some representatives of each group presented their professional approach to this problem. Posteriorly the moderator opened a panel discussion, with a starting question focused on "technology push vs. market pull as forces of innovation in this field". The central message was "the need to progress on the use of the knowledge already generated with the aim of minimizing the repercussions that parasites in general have on consumers and seafood industry". More specifically, the matter that was discussed in more detail was "anisakids", firstly due to their recognition by the European Food Safety Authority as the only family of parasites that potentially causes allergic reactions in humans, and secondly by reason of the rejections caused in consumers since it can be sometimes easily detected macroscopically.

2.3. RESULTS AND DISCUSSION

2.3.1. Maps

Epidemiological maps of *Anisakis* spp. created on the basis of the available scientific literature, shows a wide distribution of this "species complex" spreading throughout the Atlantic Ocean, even though the

sampling effortwas not equitable in whole Atlantic area, neither for all species. However, a number of "hot spots" can be identified, particularly in the Northeast Atlantic, South Africa and South America. Furthermore, distribution of marine helminth parasites can be influenced by a wide range of abiotic factors, as well as by a trophic relationship between final, intermediate and transport hosts (Kuhn, García-Màrquez, & Klimpel, 2011), a fact which may complicate the predictive mapping on infection parameters concerning commercial fish species. Despite this, the developed maps constitute a prospective valuable tool since they provide an overview of anisakids distribution and its incidence in major fish stocks. Although the impact of the epidemiological dynamics of *Anisakis* spp. on marine trophic structures and in fish populations are the subject of intensive studies, the spatial epidemiology of this re-emergent marine parasite with zoonotic and economic relevance have been disregarded so far. Nowadays, this useful tool brings important improvements to researches in several fields as medicine, health or environmental sciences. The creation of risk maps may help to underline hot-spot infection areas, as a pre-harvest control measure to reduce or minimize the risk of anisakids infection during the value chain of fishery products.

2.3.2. Inquiries

Among the 108 total surveys, 98 were performed in market squares. From them, 68 interviews (60% from the total) were conducted in cities and other 30 (28%) in villages (Figure 2.3.1). With the aim of finding out the most important aspects of concern to fish sellers in order to improve sanitary and quality condition of seafood, we asked them about the changes they would apply at their workplaces. Around the 30% of survey respondents considered that they have optimal conditions and no changes must be done, despite the lack of hot potable water for cleaning, flake ice machine, adequate refrigerators (in size and quality), or sometimes the need of an improved cleaning, which are essential aspects to ensure a proper management of commercial and sanitary quality of seafood. Furthermore, other less related or more commercial contributions like having a rainwater system with timer, better illumination over the desk, improvements in the building and in the stands, or some advances in marketing and promotion (the last two improvements were not reflected in the graphic) were proposed by them as some necessary changes in the points of sale (Figure 2.3.2). Concerning the practice of evisceration or removing specific parts of certain fish species before placing them for sale, about 17% of sellers confirmed the practice of evisceration in the case of *Pollachius pollachius*, and 6% in the case of *Trisopterus luscus*. For *M. merluccius*, 8% of responders declared to eviscerate the fish and 3% said they removed the fish hypaxial muscle (Figure 2.3.3), due to the fact that hypaxial muscle and viscera are the anatomical regions with higher amounts of larvae in parasitized fishes. Fish species with absence (*S. pilchardus*, *Z. faber*, *S. scombrus*, *Lophius* spp., *M. poutassou* and *E. encrasicholus*) or with lower (*C. conger*, *Lepidorhombus* spp., *Trachurus* spp., *Gadus morhua* and *Thunnus* spp.) percentages of evisceration and/or hypaxial muscle removing were not represented in graphics. A similar question about eviscerating and removing the hypaxial muscle before keeping fishes overnight was made. About eviscerating 13% of responders confirmed the practice, 28% performed evisceration only for

certain species, and the remained 59% did not manipulate the fish. Moreover, no more than 9% of sellers responded that sometimes remove the hypaxial muscle, depending on the species (Figure 2.3.4). The majority answered, "yes" to the question of whether they knew anisakid worms (94% of responders) (Figure 2.3.5). Finally the two following questions dealt with fish rejections and claims caused by obvious and annoying presence of anisakids in fishes. The most remarkable data is that 50% of sellers are currently rejecting fishes (of any species), and almost 50% of them are receiving complaints from customers, due to an excessive presence of anisakids. Fish species involved in both type of incidences were represented in one single graphic, in order to compare them by descriptive analysis (Figure 2.3.6). For *Merluccius* spp. and *Trigloporus lastoviza* almost the same number of rejections were made by consumers and sellers. For *Brama brama* the number of consumers' claims was higher than the amount of sellers' refusals. For *M. poutassou*, the quantity of both kinds of refusals was exactly the same. For other species included in this point of the surveys there was no coincidence between rejections and claims; so they have not been represented in the graph.

Table 2.1. Spearman rank order correlations between sellers' rejections and consumers' claims due to infection by anisakids in commercial fish species.

Fish species	***N***	***r***	***t (N-2)***	***p-level***
Merluccius merluccius	108	0.166583	1.60274	0.112495
Brama brama	108	0.292306	2.89971	0.004693
Trigloporus lastoviza	108	0.699164	9.27722	0.000000
Micromesistius poutassou	108	0.864426	16.31130	0.000000

Moreover as Table 2.1 shows, the analysis by Spearman Rank Order Correlations revealed that the relationship between refusals led by sellers and consumers' complaints in the species represented in Figure 2.3.6, was evident (r = 0.2861; p = 0.0026). Specifically, for *T. lastoviza r* value was 0.699, for *B. brama r* = 0.292 and for *M. poutassou* the correlation between refusals and complaints was the highest, giving a significant value of r = 0.864. However, for *M. merluccius* the correlation was not significant. Despite this species gave the highest number of customers' claims due to the massive presence of anisakids, fish sellers believe that there are two types of Atlantic hake; the one which comes from nearby waters ("high quality" Hake), and other from distant waters ("very parasitized" Hake). From this point, they associate Hake consumers' claims to a cause related to origin, rather than to species.

After talking with respondents it could be established that: (1) the main reason why there is a positive relationship between these two variables is because sellers usually reject fish species that generate customers complaints due to an evident presence of anisakids; (2) the fact that a fish species is highly parasitized do not lead sellers to consider it as a product unfit for human consumption, if that species can be sold eviscerated or without specific parts of musculature (more parasitized); (3) sellers are putting in practice reactive measures instead proactive actions, which would lead to better results. These facts suggest a lack of sanitary education among fish sellers. The need of training and inform more acutely for

this guild is very important since sellers are representing the sector, and have the opportunity to sensitize consumers on good management and consumption practices.

2.3.3. Round tables

During the Symposium and round tables all horizon scanners agreed that the status quo to manage the parasite hazard in the production-to-consumption food pathway is clearly unsatisfactory. They also emphasized the advantages of the long-term view of risk uncertainties associated to biological hazards for anticipating the problem and its handling. As the European Food Safety Authority, EFSA (2010) recently explained in the scientific opinion on risk assessment of parasites in fishery products, the horizon scanning is becoming a major strand in proactive risk management and patient-consumer protection continuity. Lastly, agents showed much concern for commercial rejections, their consequential economic losses and the increasing lack of confidence that anisakids and many other different types of parasites present in fishery products are currently producing.

Half a dozen of key issues to conduct research, to inform policy and to practice were specifically identified by stake-holders during the round tables:

2.3.3.1. Standardization

The lack of standardization is one of the most concerned bottleneck problems during parasite inspection in the fish industry. Improvement plans would require the development of more efficient, low cost, quick and accurate validated methods of parasite examination and detection during fish inspections. That lack of a golden standardization for fast and easy detection methods is hampering the consensus of parasite detection and diagnosis protocols at the fishing industry, thus reducing customer confidence in market transactions. The most debatable issue was the subjectivity and ambiguity of some concepts defined by legislation such as “visible parasite”, “clearly contaminated” and “obviously infested with parasites”, as specified in the European Hygiene Package (Council Directive 91/493/EEC; Commission Decision 93/140/EEC; Regulations (EC) 852-854/2004, Council Regulation (EC) 2406/96; Commission Regulation (EC) 2074/2005) and in its modifications (Commission Regulations (EC) 1662-1664/2006). These concepts evidence a lack of standard settings regarding the “*quantum satis*” conception, because no limit is defined between zero risk vs. tolerable risk. Therefore, a detection limit provided by sanitary authorities for an allowable number of larvae or amount of DNA-antigen traces in fresh fish musculature is desirable (Pascual et al., 2010). Furthermore, the accuracy of a “visual examination” scheme in the fish industry depends on the training and skills of inspectors (Levsen et al., 2005), but mostly on a well-tested statistical significance between the number of observable parasites in the abdominal cavity and surrounded organs, and the number of parasites in musculature (Llarena-Reino et al., 2012). Although this method does not guarantee a parasite-free edible part of fish, no other method as a golden standardization has been accepted as the international reference protocol accomplish with the industrial requirements. Moreover, the establishment

of epidemiological monitoring programmes to standardize the methodology for fish inspections should comprise the definition of the concepts “sampling size” or “epidemiological unit” which are not defined by legislation. These issues represent a source for uncertainty in hazard analysis during fish safety and quality self controls.

2.3.3.2. Monitoring

As most of scanners stated the industry as responsible of food security and quality, needs tools to detect parasites, sanitize seafood products and develop effective management strategies. They proposed that proactive self-inspections carried out by fish operators could provide a chance to transform the parataxonomic inspection carried out by the industry into a zoosanitary vigilance program by networking an industrial upgrading of national sanitary defense associations, as it is the case in aquaculture production. Furthermore, it also would be advisable to take into account samples from oceanographic and evaluation resource campaigns financed by national governments and international funds, which periodically are operated by research entities.

2.3.3.3. Innovation

Group discussion proposed to invigorate collaborative translational research and professional training as key drivers to fuel technological innovations and tech transfer, which may help to minimize or eliminate the risk of parasites with public health and/or economic concerns in fish products. With the increasing demand for ready-to-eat, fresh, and minimally processed fish, new ecology routes for parasite survival have emerged as it was demonstrated in modified atmosphere packaging (Pascual et al., 2010). In order to minimize the loss of quality and to control parasite hazard, hurdle technology was suggested in the design of preservation systems for minimally processed foods at various stages of the food chain. However these new and other emergent technologies such as ultrasounds, electrolyzed oxidizing water, etc., should be specifically evaluated for parasite hazards. Additionally, the proportionality of innovations that take into account the weight up of cost-benefit ratios for different interventions in the food chain was also stressed by industrial stake-holders. Finally, they also identified technological and economic benefits in outsourcing R&D in an open innovation strategy for component improvements, design and new process/product innovations.

2.3.3.4. Training

In general all fish food industry employees in Europe are educated and trained in relevant food safety practices, beyond basic food handler training. Some available guidebooks describe the good manufacturing practices and safe fish handling procedures that help fishermen, fish processors, truckers and retailers to assure and maintain the food safety and fish products quality from the boat to the retail counter. Nevertheless, educational seminars for relevant emerging topics like parasite hazards are needed and are

still absent in many European regions. As surveys revealed, there is lack of sanitary education concerning parasites among fish sellers; they confuse basic notions and are not able to differentiate those parasites which can cause zoonotic disease, from those innocuous to public health.

2.3.3.5. Risk assessment

Among the surveys' findings, it was noted that fish sellers' rejections due to excessive parasitism matched in amount and fish species with consumers' complaints. Repeatedly, sellers' criteria seem to be conditioned by consumers' reactions to parasites. That absence of a proactive behavior at points of sale implies that prevention is not being applied. Much more risk assessment information, both in fish products and for consumers and sellers has been a relevant plea throughout horizon scanning round tables. A friendly SMART (self-monitoring and intelligence reporting technology) platform has been suggested to generate pre-harvest control tools (e.g., risk maps and epidemiological reporting). The design of methodologies of categorization or staging which should include the parasite identity, the spread of parasites in the edible part of fish, and the food quality and safety implications of this biological hazard, were also recommended. The development of this kind of risk-based metrics (point and probabilistic estimates) should be incorporated, implemented and monitored in HACCP plans. Risk assessment from a public health perspective demands relationships between catch origins, fish species, fish stock structure and parasite quantitative descriptors, in different "what-if" and scenarios for parasite animals, traces and antigens. Its purpose is to attend natural variability and scientific uncertainty through statistical inference. Mapping of *Anisakis* allergens in seafood and a deeper understanding of immune response to the parasite antigens were noted as important tasks for research. Furthermore, integration of epidemiological information on infectivity and inactivation of parasites taking the whole production-to-consumption food pathway, and the incidence of this zoonotic infection in humans, will aid to analyze, predict and prevent the probability of illness, complaints and fish rejections, thus enhancing public awareness and the effectiveness of control measures. As one of the more strong initiatives, stake-holders also proposed to create and develop a thematic network performance based on collaborative software to provide multi-level information (on-site and at-line) for industrial management of parasite contaminants in fish products. The ultimate goal for all implicated horizon scanners during this event was the collaboration and the establishment of common spaces between agents, industries and scientists, getting thereby better advances in the strategies and technologies to fight against this important hazard. Only by achieving this purpose the international competitiveness of fish products could be enhanced.

2.3.3.6. Risk communication

Risk communication was determined by scanners as a matter of concern to manage alerts instead of alarms. It was suggested to elaborate a risk profile for each emergent parasite species with the aim of sharing multi-level information and to aid technology-knowledge transfer. Each "parasite array" will assure

communication with public regulatory authorities and the industry, thus reinforcing the industry's competitiveness by implementing added value strategies to guarantee a high standard quality in healthy fishery products. Similarly to the above knowledge-based bio-economic approach, it would be of high priority to spread the knowledge to the broader society to ensure consumer protection within an open public access plan. To be relevant and useful the participants agreed to bring horizon scanning under a QCA perspective by repeating the process and collation annually, and to include the topic and the information in the working groups of the European Fish Technology Platform.

2.4. CONCLUSIONS

The data collected from the maps, inquiries and during the round tables contain valuable suggestions orienting current and future strategies, identifying key problems with the existing procedures and providing advices that could improve public health policy and reduce economic losses. These ideas have been summarized and compiled around six key issues comprising a very constructive horizon scanning effort for managing emerging parasites in fishery products, as follows:

- The lack of standardization during parasite inspection in the fish industry is the main reason why the industry demands that the transfer of food safety co-responsibility from governments to companies should be led by a tough and progressive program of unified standards more closely monitored by governments. This lack of consensus and standardization concerning self-control, makes easier a free criteria and heterogeneity when internal inspection of batches, manufacturing facilities or processes take place. FAO protocols, facto standards by Codex Alimentarius, military standards or statistical standards are some examples of quality criteria in use for internal controls by food companies.

- Supervised proactive self-inspections at industries could lead to set up stable zoosanitary vigilance programs. The monitoring of demographic values of infection by parasites in fishes could be integrated for its study as a part of the evaluation programs during oceanographic campaigns.

- The setting of innovations based in positive weight up of cost-benefit ratios as labeling requirements for parasite-free trademarks, could provide a chance for enable commercial blister beneficiaries of process monitoring programs, for periodic analysis of products and for preventive and corrective measures for parasites with public health and economic implications. Furthermore, the elaboration of an innovation guide directory with the portfolio of services was suggested as a key drive to help identify organizations which do outsourcing R&D work for fish companies.

- Educational seminars concerning relevant emerging topics like parasite hazards, for industry employees and retailers should be implemented in all European regions, especially the establishment of proof-of-concepts and demos linked to GMP and SOP programs within the legal scenarios to

monitor into real-life. Fish sellers represent a critical point that must be conscientiously trained and instructed, since they are the target group to reach the consumer through an immediate, inexpensive, effective, continuous and conservative approach.

- Regardless of the method used for fish inspection, it is essential to design methodologies of categorization or staging which should be incorporated, implemented and monitored in HACCP plans. Integration of epidemiological information of parasites will aid to study, predict and avoid fish rejections and zoonoses, and will enhance public consciousness and the success of control measures.

- With the aim of improving risk communication to the broader society it would be indispensable to spread the knowledge to ensure consumer protection within an open public access plan.

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CHAPTER 3

Diagnostic Methods (I)

The accuracy of visual inspection

Llarena-Reino, M., González, Á.F., Vello, C., Outeiriño, L. and Pascual, S. (2012). The accuracy of visual inspection for preventing risk of *Anisakis* spp. infection in unprocessed fish. Food Control, 23(1):54-58.

ABSTRACT

The importance of the zoonoses caused by L3 Anisakidae larvae lies in the repercussion that this parasite exerts on food safety and quality. EU legislation recommends fish operators to do visual inspection of the whole fish abdominal cavity and gut to control the risk of visible parasites, thus ensuring that no contaminated fish reach the consumers. The accuracy of the above visual inspection method should fall on a well-tested statistical significance between the number of observable parasites in the abdominal cavity and the number of parasites in the edible part of the fish (i.e., musculature). The aim of this study was to analyse this statistical significance, and the efficacy of the washing practice to remove *Anisakis* spp. from gut. To carry out this work, 322 fresh individuals of *Micromesistius poutassou* and 230 of *Scomber scombrus* were necropsied within 12 hours and 48 hours post-capture. Then, descriptive statistics, correlation and regression analyses were used to evaluate the significant statistical relationship between the number of anisakid larvae found in the gut and musculature of both fish species. Additionally, livers and gonads of 25 fresh specimens of *Merluccius merluccius* were vigorously washed under tap water, and examined under stereomicroscope looking for *Anisakis* spp. larvae. Results evidenced the low efficiency of visual inspection of gut parasites as a commonly recommended method for predicting nematode larvae in the flesh of fish. Therefore, a direct-invasive inspection of musculature is stressed as the only criteria with scientific merit for accurately detecting contaminated fishes by anisakids. Moreover, fresh European hake liver and gonads showed at least one larva remained inside the tissue after washing vigorously under tap water. Results suggested that critical control points at Hazard Analysis Critical Control Point (HACCP) programmes should be reviewed to improve the risk of anisakid-induced allergies and gastrointestinal anisakiasis among consumers.

KEYWORDS

Anisakis spp. larvae; fish; gut; musculature; parasites; significant statistical relationship

3.1. INTRODUCTION

Anisakids are marine cosmopolitan parasites highly prevalent in wild fish stocks of commercial interest species. They are usually found in high amount in the third larval stage on the gut cavity and sometimes on the belly flaps too, during fish inspections (Abollo et al., 2001). These parasites are recognized as human health hazard responsible for emergent zoonoses called anisakiasis, causing gastro-allergic disorders in consumers and occupathional-asma in fish-farming workers (Plessis et al., 2004; Nieuwenhuizen et al., 2006).

In the transborder Euroregion Eixo Atlantico (NW Iberian Peninsula), the traditional *escandallo* or inspection procedure, is a rapid and reliable sensory method largely used in the seafood industry to ensure the quality of fishery products and to make commercial trade more confident. The above inspection method follows an internationally used protocol which should guarantee the safety of inspected seafood products. In fact, at the Euroregion, some international companies inspect and evaluate the risk of these biological contaminants by managing these inspections in retail chains, certifying customers that no prohibited contaminants are in fact present at the critical control points from the fishery to the plate. EU legislation (Commission Regulation (EC) 2074/2005; (EC) 853/2004 rev.) pointed out that visual inspection of the whole fish abdominal cavity (including liver, gonad and egg mass) should be done by fish operators to control the risk of visible parasites, thus ensuring from the catch to the plate that no contaminated fish reach the consumer.

The accuracy of a visual inspection method in the fish industry largely depends on the training and skills of inspectors (Levsen et al., 2005), but mostly on a well-tested statistical significance between the number of observable parasites free or encysted in the abdominal cavity and surrounded organs, and the number of parasites in musculature or edible part of the fish. The later is especially important when expending untreated fresh fish products (e.g., coastal fish), because no prophylactic processes have been carried out to kill *Anisakis* spp. larvae or inactivate their somatic and metabolic antigens during harvest and distribution, making the final consumer manage the hazard.

The double aim of this work was (1) to study the existence of a statistical significance between gut parasites and muscular parasites, and (2) to evaluate the efficiency of the washing practice to remove *Anisakis* spp. from gut, in order to evaluate the accuracy of the current legislation.

3.2. MATERIALS AND METHODS

Commercial lots of 322 fresh individuals of the blue whiting *Micromesistius poutassou* and 230 of Atlantic mackerel *Scomber scombrus,* caught in the western Iberian Sea (ICES division IXa), were necropsied within 12 hours and 48 hours post-capture. The time passed after capture, the number of fishes in each lot and the ranges of total length and total weight for both species are showed in Table 3.1.

Table 3.1. Biological data as host sample size (N), time between capture and necropsies, and total length and weight ranges of the fish species studied for *Anisakis* spp. infection.

Species	***(N)***	***Hours Post-capture***	***Total Length Range (cm)***	***Total Weight Range (g)***
Micromesistius poutassou	163	12	21.5-28	68-172
Micromesistius poutassou	166	48	21-28.5	52-158
Scomber scombrus	166	12	27-34	123-291
Scomber scombrus	70	48	31-43	204-645

The heads and tails were removed from each fish, and the remaining musculature was separated into the hypaxial (ventral) and epaxial (dorsal) regions following the horizontal septum. The nematodes were isolated by digestion from the whole gut and from the fish musculature, according to CODEX STAN 244-2004 rev. Sixteen variables were recognized and defined to compare the number of *Anisakis* spp. larvae, taking into account fish species, fish body region and time from capture to necropsies (Table 3.2).

Table 3.2. Sixteen variables have been established to compare A*nisakis* spp. larvae at the study, taking into account fish species, fish body region and time from capture to examination.

Variable	***Species***	***Body Region***	***Hours Post-capture***
MPH12	*Micromesistius poutassou*	Hypaxial Musculature	12
MPE12	*Micromesistius poutassou*	Epaxial Musculature	12
MPT12	*Micromesistius poutassou*	Hypaxial and Epaxial Musculature	12
MPG12	*Micromesistius poutassou*	Gut Cavity	12
MPH48	*Micromesistius poutassou*	Hypaxial Musculature	48
MPE48	*Micromesistius poutassou*	Epaxial Musculature	48
MPT48	*Micromesistius poutassou*	Hypaxial and Epaxial Musculature	48
MPG48	*Micromesistius poutassou*	Gut Cavity	48
SSH12	*Scomber scombrus*	Hypaxial Musculature	12
SSE12	*Scomber scombrus*	Epaxial Musculature	12
SST12	*Scomber scombrus*	Hypaxial and Epaxial Musculature	12
SSG12	*Scomber scombrus*	Gut Cavity	12
SSH48	*Scomber scombrus*	Hypaxial Musculature	48
SSE48	*Scomber scombrus*	Epaxial Musculature	48
SST48	*Scomber scombrus*	Hypaxial and Epaxial Musculature	48
SSG48	*Scomber scombrus*	Gut Cavity	48

Descriptive statistics for parasite counts including the mean, median, mode, variance, skewness, kurtosis, a box-whisker graph and a Kolmogorov-Smirnov test were calculated. Correlation and regression analyses, were also used to evaluate the significant statistical relationship between variables, regarding the number of *Anisakis* spp. larvae found in the gut and musculature (epaxial, hypaxial and total musculature, separately) of both fish species. Spearman correlation coefficient (*r*), t (N-2) and *p*-level values (for statistical significance) only were specified for pairs of variables which revealed correlation between variables. When necessary, anisakid counts were logarithmic transformed to normalize the data (Rózsa et al., 2000).

Moreover, demographic values of infection for Anisakis spp. larvae were determined specifically for gut, epaxial and hypaxial region, and total musculature at both fish species. The terms prevalence, mean intensity and mean abundance of infection were used as defined in Bush et al. (1997) and Rózsa et al. (2000).

Additionally, a commercial lot of 25 fresh individuals (250-300 mm sized) of the European Hake *Merluccius merluccius,* was necropsied 12 hours post-capture. Fresh liver and gonads were vigorously washed under

tap water. Then, both organs were examined under stereomicroscope looking for the presence of *Anisakis* spp. larvae, and infected tissues were processed for histological sections following standard protocols.

3.3. RESULTS

Descriptive statistics for anisakids counts in both fish species showed that any of the *Anisakis* spp. count combining variables did not follow a normal distribution (Kolmogorov-Smirnov Test <0.05) (Table 3.3; Figure 3.1).

Table 3.3. Demographic infection values and descriptive statistics for anisakids counts in defined variables.

Variable	*N*	*Prevalence (% ± CI)*	*Mean Intensity (± SD)*	*Mean Abundance (± SD)*	*Mean*	*Median*	*Mode*	*Variance*	*Skewness*	*Kurtosis*
MPG12	163	94.47 ± 1.75	12.18 ± 14.47	11.5 ± 14.34	11.5092	7.00	3.00	205.745	3.106	12.616
MPE12	163	4.3 ± 1.5	1.71 ± 0	0.07 ± 0.16	0.02454	0.00	0.00	0.024	6.203	36.935
MPH12	163	33.13 ± 3.6	1.77 ± 1.23	0.59 ± 1	0.51534	0.00	0.00	1.078	3.055	11.216
MPT12	163	34.97 ± 3.6	1.89 ± 1.23	0.66 ± 1.04	0.53988	0.00	0.00	1.077	2.995	10.957
MPG48	166	98.79 ± 0.83	69.18 ± 92.48	68.35 ± 92.23	68.3494	40.50	31.00	8506.398	4.195	24.369
MPE48	166	12.05 ± 2.4	2.05 ± 1.28	0.25 ± 0.91	0.23494	0.00	0.00	0.835	5.638	37.846
MPH48	166	75.3 ± 3.29	7.02 ± 13.07	5.29 ± 11.72	5.24096	2.00	0.00	137.432	6.467	55.758
MPT48	166	76.5 ± 3.22	7.24 ± 13.32	5.54 ± 12.03	5.4759	2.00	0.00	144.784	6.182	51.559
SSG12	166	72.89 ± 3.38	11.55 ± 51.77	8.42 ± 44.45	8.42169	2.00	0.00	1975.942	12.007	150.355
SSE12	166	1.2 ± 0.82	1 ± 0	0.01 ± 0.11	0.01205	0.00	0.00	0.012	9.027	80.451
SSH12	166	18.67 ± 2.96	2.16 ± 1.92	0.4 ± 1.16	0.38554	0.00	0.00	1.341	4.488	24.136
SST12	166	19.28 ± 3	2.16 ± 1.96	0.42 ± 1.19	0.40361	0.00	0.00	1.418	4.297	21.786
SSG48	70	57.14 ± 5.79	4.92 ± 9.58	2.81 ± 7.61	2.81429	1.00	0.00	57.893	7.048	54.818
SSE48	70	5.7 ± 2.7	1 ± 0	0.06 ± 0.23	0.05714	0.00	0.00	0.055	3.899	13.597
SSH48	70	34.3 ± 5.54	2.25 ± 3.23	0.77 ± 2.08	0.72857	0.00	0.00	4.346	4.869	26.491
SST48	70	38.57 ± 5.7	2.25 ± 3.04	0.83 ± 2.08	0.78571	0.00	0.00	4.345	4.791	25.942

Table 3.3 shows demographic values (prevalence, mean intensity and mean abundance) of infection attributable to *Anisakis* spp. larvae, calculated specifically for gut, epaxial and hypaxial region, and total musculature at both fish species. These values clearly evidenced higher infection in gut than in musculature, and larger values of worm burdens in hypaxial region than in epaxial musculature, in all cases. Other results in the same table suggested that an increased mean, median and variance of *Anisakis* spp. larvae in the gut of *Micromesistius poutassou* at 48 hours post-capture led increments in the mean, median and variance of these parasites in the hypaxial region and at the total musculature in the same group of fishes. This tendency was not observed in the rest of the fish lots analized (*Micromesistius poutassou* at 12h, *Scomber* scombrus at 48h and at 12h).

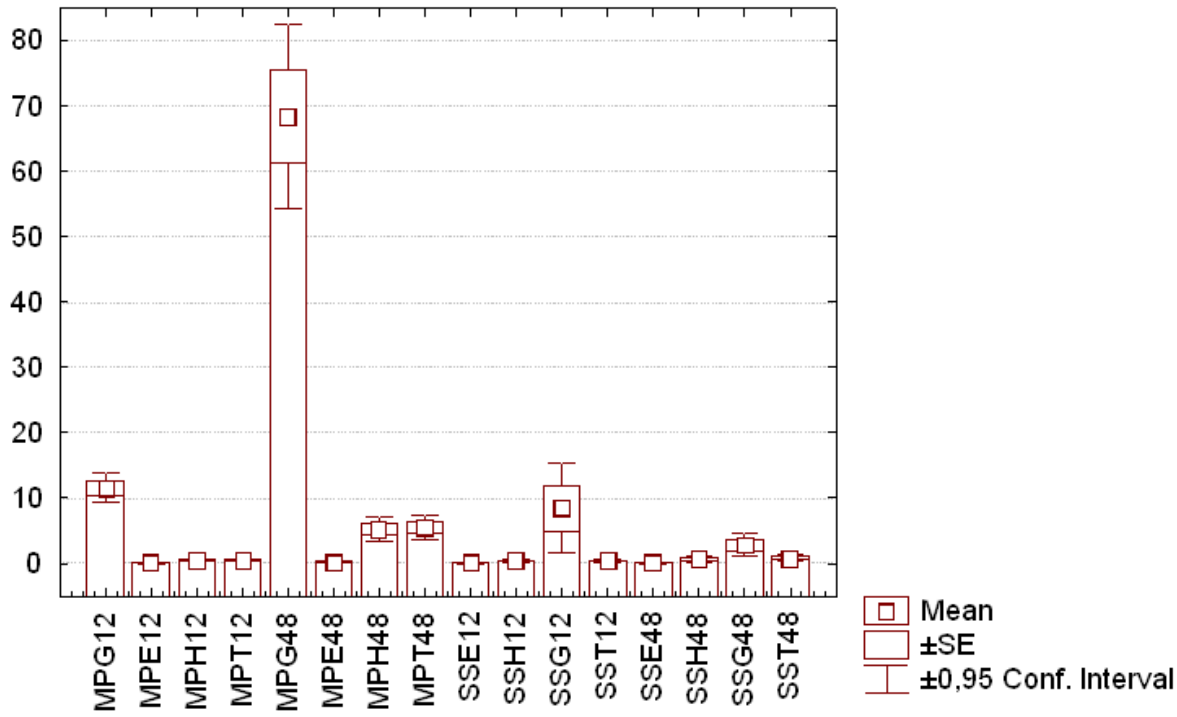

Figure 3.1. Box-whisker graph of anisakid counts in fish gut and musculature (epaxial, hypaxial and total). The number of *Anisakis* spp. larvae (vertical axis) is represented for each variable defined and studied (horizontal axis).

Every pairs of variables were analysed by Spearman Rank Order Correlations (Table 3.4). The results revealed that the worm burden in the total musculature was more correlated to the parasites present at hypaxial musculature (*r* values between 0.92-0.98) than at epaxial region, which gaves lower significant rates (*r* =0.18-0.38) at 12h and 48h post-capture in both fish species. Moreover, there was a positive relationship (*r*=0.25-0.51) between gut and total muscular worm burdens at *Micromesistius poutassou* at 48h and *Scomber scombrus* at 12h and at 48h. The positive relationship between gut and musculature in *Micromesistius poutassou* at 48h was significantly higher specifically at hypaxial muscular region (*r*=0.52) than at epaxial muscle (*r*=0.21). As well for *Scomber scombrus* at 12h, the same positive relationship was higher at hypaxial muscle (*r*=0.34) than at epaxial (no significant correlation). However, *Scomber scombrus* at 48h did not give interesting values of correlation between anisakids in gut and hypaxial or epaxial musculature. These were two of the eight remaining pairs (including all variables not showed in Table 3.4) that presented an absence of strength between the variables compared in each pair (at $p<0.05$). This fact also occurred, for example, when comparing the number of parasites in the gut of blue whiting, with the parasites in the musculature (any of regions) at 12 hours post-capture. Even the number of parasites at both regions of the musculature had no correlation between them. Equally, Atlantic mackerel at 48 hours post-capture showed no associations in the number of parasites comparing epaxial and hypaxial musculature.

Table 3.4. Spearman Rank Order Correlations between variables. Spearman correlation coefficient (r) and p-level (value of the statistical significance at 0.05) are given for pairs of variables which present correlation. Pairs without some intensity of correlation have not been taken into consideration.

Pair of variables	*N*	*Spearman (r)*	*t (N-2)*	*p-level*
MPE12 - MPT12	163	0.188116	2.43031	0.016185
MPH12 - MPT12	163	0.956773	41.74187	0.000000
MPG48 - MPE48	166	0.211691	2.77384	0.006182
MPG48 - MPH48	166	0.527729	7.95636	0.000000
MPG48 - MPT48	166	0.512729	7.64793	0.000000
MPE48 - MPH48	166	0.292033	3.91030	0.000135
MPE48 - MPT48	166	0.380792	5.27385	0.000000
MPH48 - MPT48	166	0.988358	83.18953	0.000000
SSE12 - SSH12	166	0.261501	3.46958	0.000666
SSE12 - SST12	166	0.263223	3.49412	0.000612
SSH12 - SSG12	166	0.343702	4.68707	0.000006
SSH12 - SST12	166	0.982838	68.23008	0.000000
SSG12 - SST12	166	0.349530	4.77751	0.000004
SSE48 - SST48	70	0.312904	2.71669	0.008355
SSH48 - SST48	70	0.926179	20.25402	0.000000
SSG48 - SST48	70	0.258036	2.20241	0.031030

Simple linear regression analysis of gut vs. muscular anisakids for both species, showed no significant relationship between the number of parasites in the gut cavity and those in any other region of the musculature (Table 3.5). This absence of statistical significance was the observed pattern every case, except for the SSG48 - SST48 pair, the only one that evidenced a causal relationship between them.

Table 3.5. Statistics of simple linear regression of gut vs. muscular (epaxial, hypaxial and total) parasites using log-transformed data. F (test for statistical significance of the regression equation), p-level (value of the statistical significance at 0.05) and the coefficient of determination R2, are represented for *Micromesistius poutassou* and *Scomber scombrus.*

	Micromesistius poutassou						***Scomber scombrus***					
	Epaxial		**Hypaxial**		**Total**		**Epaxial**		**Hypaxial**		**Total**	
	12h	**48h**	**12h**	**48h**	**12h**	**48h**	**12h**	**48h**	**12h**	**48h**	**12h**	**48h**
F	0.074	0.292	0.029	0.580	1.096	0.491	0.000	1.099	0.028	0.009	0.028	5.778
***p*-level**	0.785	0.600	0.865	0.447	0.297	0.484	0.992	0.298	0.867	0.924	0.866	0.019
R^2	-	-	-	-	-	-	-	-	-	-	-	0.079

Otherwise, examination of liver and gonads from fresh European hake showed high demographic values of Anisakis spp. infection (Table 3.6; Figure 3.2.A-C).

Table 3.6. Infection values for *Anisakis* spp. in the gonads and livers of European hake *Merluccius merluccius*.

	Merluccius merluccius	
	Gonads	**Liver**
N	25	25
Prevalence (% ± CI)	0.64 ± 0.13	0.84 ± 0.10
Mean Intensity	9.2	21.23
Mean Abundance	6.1	17.84

After washing vigorously under tap water most *Anisakis* spp. larvae were removed but in all cases at least one larva remained inside the tissue. These larvae usually corresponded with deeply embedded parasites or older capsules that were observed in histological sections (Figure 3.2.D-F).

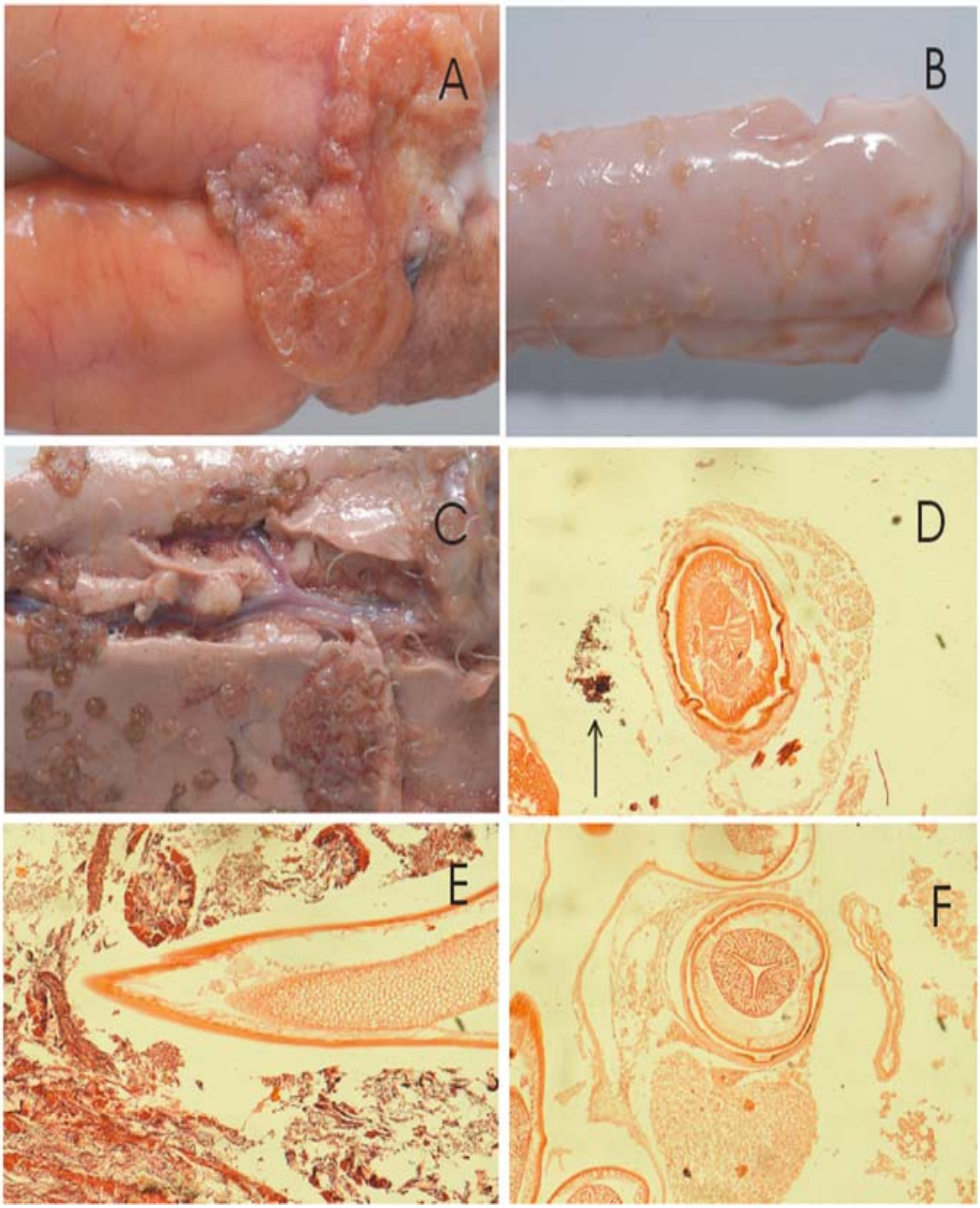

Figure 3.2. (A-F). Macrophotograps and histological sections stained with hematoxylin and eosin (40X) of liver and gonads heavily infected with *Anisakis* spp. larvae. **A-C:** The parasites are located encysted inside gonads (A and B) and liver (C), as well as covering them. **D-F:** Cross-section of an embedded larva inside the female reproductive tract (D). Black arrow: Rests of an old capsule (melanin granules) surrounding the parasite. Longitudinal section of an embedded larval inside the male reproductive tract (E). Cross-section of four embedded larvae inside the liver (F).

3.4. DISCUSSION

Results suggested the low efficiency of visual inspection of gut parasites as a commonly recommended method for predicting nematode larvae in the flesh of fish. In fact, association does not imply predictability. It is feasible that by counting many parasites in gut someone can have an idea that many parasites are in fact infecting the fish musculature, but it is not easy to predict how many parasites will be found there. This implies that in absence of anything better for fish operators, correlation matrices are useful but not enough to ensure a robust statistical predictable value to infer muscular anisakids based on the evidence of gut parasites. This is the case of blue whiting, which none significant relationships between parasites in gut and flesh regions was determined, after linear regression analyses. Furthermore, in the best case (e.g., in the Atlantic mackerel inspected at 48 hours) the amount of variability in the dependent variable, *number of muscular parasites,* explained by the predictor variable, *number of gut parasites,* was less than 8% (as estimated by the R^2). Bussmann and Ehrich (1979) studied blue whiting as well, from different geographical sampling areas and seasons. He reported linear regression analyses with significant positive associations (p<0.05) between the number of parasites in gut, hypaxial musculature and epaxial flesh, based in not normalized data. However, as some other authors recommends, raw data of the frequency distribution do not work well, and a good alternative to proceed is the log transformation (log[x+1]) before calculating the mean (Rózsa et al., 2000). In addition, different geographical sampling areas and seasons could influence on relationships between sites of infestation (Bussmann and Ehrich, 1979).

In relation to demographic values of infection obtained from the biological data, comparing prevalences at both species with the same hours post-capture, higher percentages of parasites in blue whiting than in Atlantic mackerel were noticed (for 12h and 48h post-capture). Mean intensity comparisons revealed four clearly different degrees of infection. At least for the four main groups of fishes that this study revised, the order of the regions according to their degree of infection (from highest to lowest) coincided the same; (1) gut cavity, (2) total musculature, (3) hypaxial musculature and (4) epaxial musculature. In all cases, mean intensity of hypaxial muscles influenced very strongly on total musculature. The highest values of *Anisakis* spp. larvae in hypaxial or in total musculature were obtained at the group with the highest worm burden value in gut (*Micromesistius poutassou* at 48h post-capture). At the same time, the lowest intensity of worms at epaxial region was found at the group with the lowest number of parasites in gut. Both facts may have been due to three factors: the distance from epaxial region to gut, the proximity of hypaxial

musculature to gut, and the larvae migration that can occurs *intra-vitam* or subsequently to host death. Many factors can explain the possibility and timing (*intra-vitam* or *post-mortem*) of anisakid migrations from fish gut to the flesh, mostly related to physiological trade-off of parasites, to ecological and immunological factors operating in living fish, or to the biochemical *post-mortem* changes which occurred in autolysed fish (Karl, 2008). Recently, Scientific Opinion on risk assessment of parasites in fishery products by the Panel on Biological Hazards of the European Food Safety Authority (EFSA, 2010) stated that "based on scientific evidences it is not clear when, under what conditions and in which fish species, *post-mortem* migration of *A. simplex* larvae occurs". In summary, these appreciations evidence different proportions of infection that can be found in fishes depending on the anatomical region. But these proportions that may be considered as "stages of infection" can fluctuate more or less, if comparing different fish species. The observation of both types of parasites (*intra-vitam* and *post-mortem*) inhabiting fish musculature, emphasized that in case of significant regression values for a given fish species, the predictive model only would be workable to infer muscular anisakids in fish inspections, if preliminary epidemiological data for that target commercial fish are available. These data would provide the penetration rate (the ratio of the number of larvae detected in the muscle to the total number of larvae detected under various holding conditions), from the abdominal cavity into the muscle of the fish. The establishment of this epidemiological monitoring programme would also allow the standardization of inspection methodology including sampling size in each commercial species, following the current artificial digestion protocol by CODEX STAN 244-2004 rev. These issues are not defined in legislation and represent a source for uncertainty in hazard analysis during fish inspections. Moreover, other edible fish parts such as gonads and liver remain contaminated with *Anisakis* spp. after gutting and washing gut vigorously under tap water which clearly does not accomplish with legislation.

The above information should be taking into account to review critical control points at HACCP programmes to reduce the risk of anisakid-induced allergies and gastrointestinal anisakiasis among consumers. This is especially important for whole ungutted fish at local markets of the Euroregion which are stowed refrigerated and sold at the market up to 2-3 days post-capture.

3.5. ACKNOWLEDGEMENTS

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CHAPTER 4

Diagnostic Methods (II)

Optimization of the pepsin digestion method

Llarena-Reino, M., Piñeiro, C., Antonio, J., Outeiriño, L., Vello, C., González, Á.F. and Pascual, S. (2013). Optimization of the pepsin digestion method for anisakids inspection in the fishing industry. Veterinary Parasitology, 191:276-283.

ABSTRACT

During the last 50 years human anisakiasis has been rising while parasites have increased their prevalence at determined fisheries becoming an emergent major public health problem. Although artificial enzymatic digestion procedure by Codex (CODEX STAN 244-2004: Standard for salted Atlantic herring and salted sprat) is the recommended protocol for anisakids inspection, no international agreement has been achieved in veterinary and scientific digestion protocols to regulate this growing source of biological hazard in fish products. The aim of this work was to optimize the current artificial digestion protocol by Codex with the purpose of offering a faster, more useful and safer procedure for factories workers, than the current one for anisakids detection. To achieve these objectives, the existing pepsin chemicals and the conditions of the digestion method were evaluated and assayed in fresh and frozen samples, both in lean and fatty fish species. Results showed that the new digestion procedure considerably reduces the assay time, and it is more handy and efficient (the quantity of the resulting residue was considerably lower after less time) than the widely used Codex procedure. In conclusion, the new digestion method herein proposed based on liquid pepsin format is an accurate reproducible and user-friendly off-site tool, that can be useful in the implementation of screening programs for the prevention of human anisakiasis (and associated gastroallergic disorders) due to the consumption of raw or undercooked contaminated seafood products.

KEYWORDS

Anisakids; CODEX STAN 244-2004; digestion method; fish; liquid pepsin.

4.1. INTRODUCTION

Anisakid roundworms (*Anisakis, Contracaecum and Pseudoterranova*) are recurrently found in the abdominal cavity (including gut) and flesh of a large variety of fish and cephalopod species of commercial interest, regularly consumed by humans. The third larval stage is transmitted through the consumption of raw or minimally processed seafood, and may cause pathogenic diseases like gastric or intestinal anisakiasis (Kikuchi et al., 1990; Esteve et al., 2000; Lopez-Serrano et al., 2003; Nawa et al., 2005; Mineta et al., 2006), and gastro-allergic disorders (Alonso-Gómez et al., 2004; Plessis et al., 2004; Nieuwenhuizen et al., 2006; Audicana and Kennedy, 2008; Hochberg and Hamer, 2010). The effects of anisakids on decreasing commercial value of fish (Vidacek et al., 2009) and its impact on human health has given these parasites a public health concern, which was recently recognized by the Panel on Biological Hazards of the European Food Safety Authority (EFSA, 2010). During the last 50 years, the significance of this double effect has been growing as parasites have increased their prevalence being more relevant in North Atlantic fisheries (Smith and Wootten, 1979; McClelland et al., 1985; Adams et al., 1997; Abollo et al., 2001; Rello et al., 2009), and

due to the lack of awareness of this potential threat among consumers. Consequently, several methods have been developed for detection, diagnosis and identification of parasites in fish, from visual inspection (Hartmann and Klaus, 1988), light microscopy (Rijpstra et al., 1988), candling (Wold et al., 2001; Butt et al., 2004), pepsin digestion (Lysne et al., 1995; Lunestad, 2003; Thien et al., 2007; Thu et al., 2007), UV illumination (Adams et al., 1999; Levsen et al., 2005; Marty, 2008), ultrasound (Hafsteinsson et al., 1989; Nilsen et al., 2008), X-Rays (Nilsen et al., 2008), conductivity (Nilsen et al., 2008), electromagnetism (Haagensen et al., 1993; Choudhury and Bublitz, 1994), magnetometry (Jenks et al., 1996), immunodiagnoses (Xu et al., 2010), multilocus electrophoresis (Mattiucci et al., 1997; Abollo et al., 2001), RT-PCR (Fang et al., 2011), real-time FRET (Fluorescence Resonance Energy Transfer) (Monis et al., 2005; Intapan et al., 2008), PCR (Zhu et al., 2002; Abe et al., 2005; Pontes et al., 2005), to Imaging Spectroscopy (Heia et al., 2007). Nevertheless, although all these methods have been used and are being applied by fishery operators or laboratories as integrated strategies in official and self-control tests, none of them has been accepted as the international standard accomplishing with industrial requirements. That lack of a gold standard for any of the above given methods, mainly for a fast and easy visual detection, has historically hampered the consensus of parasite detection and diagnosis protocols at the fishing industry, thus reducing consumer confidence towards seafood companies.

Specifically, acidified pepsin solution has been largely applied as a confirmatory invasive protocol to detect absence or presence of nematodes in fish products (Lunestad, 2003), and as a tool to quantify parasitic infections and to estimate the number of parasites in the fish musculature (Lysne et al., 1995; Thien et al., 2007; Thu et al., 2007). Some additional variations of the pepsin digestion method from CODEX STAN 244-2004 protocol have been developed by some authors (CX/FFP 08/29/7; Dixon, 2006) with attempts to go further, specifically in improving the method and more widely in developing faster methodologies for biological threats detection.

According to the two definitions of "optimization" provided here (“to achieve maximum efficiency in storage capacity or time or cost” and “to make as effective, perfect, or useful as possible”), the aim of this work was to improve and optimize the current artificial digestion protocol of Codex by (1) evaluating three different brands of commercial pepsins on different fish products (e.g., lean/fatty and fresh/frozen), (2) implementing new conditions on the basis of the current digestion procedure, and (3) comparing the new practice proposed with the currently used one. As a result, a new analytical methodology is offered based on the modification of the existing artificial digestion of fish flesh provided by Codex.

4.2. MATERIALS AND METHODS

4.2.1. Samples

Fresh fishes obtained at retail both of European hake (*Merluccius merluccius*) and Atlantic mackerel (*Trachurus trachurus*), were used as representative samples of lean and fatty fish species, respectively. Half of them were processed in fresh and half were immediately frozen at -20°C for at least 24 hours, and afterwards processed. Three different commercial pepsins were preselected to be evaluated: a commonly used pepsin (pepsin 1), the recommended reagent in Codex protocol (pepsin 2) and a novel liquid format (pepsin 3). For understanding and presenting their proteolytic activities, equivalences between different units used in commercial pepsins were taking into account (Langdon, 2009). Proteolytic activities indicated by the three manufacturers for the three pepsins were: 800-2,500 Units/mg of protein, 2000 Units/g FIP (International Pharmaceutical Federation), and 660U Ph Eur (European Pharmacopeia)/ml, respectively. Authors understand that enzymatic activities specified do not need verification because it would not be viable to develop routine protocols, since it should be necessary to perform a check of any pepsin before its use. Therefore, in order to minimize any imprecision related to the reagents, all of the pepsins used in this study were acquired, stored, prepared and treated properly under the same criteria and under identical conditions (specified by manufacturers).

4.2.2. Pepsin assays

Briefly, six aliquots of 25g each from both fresh and frozen fish species were digested with the three different pepsins at 37°C during 30 minutes in an ACM-11806 Magnetic Stirrer with thermostated heating Multiplate. The weight/volume pepsin ratio used was 1:20, understanding that ratio as one gram of fish for twenty milliliters of a 0.5% pepsin solution in HCl 0.063M pH 1.5. Undigested muscle residues of each kind of fish and pepsin were weighed and compared, without taking into account the weight due to the parasites in the positive samples.

In order to compare the two pepsins that previously had given higher percentages of digested muscle, appropriate calculations were made to determine the pepsin dose necessary in each case to prepare solutions containing the same proteolytic activity. To this end, density of liquid pepsin (1.215 Kg/m3) and equivalence units previously mentioned were taken into account. Enzymatic activity was set at 5000 FIP Units/g, because this is the resultant value when applying the Codex method. One more time, six samples of 25g each of fresh hake and mackerel were digested with the two pepsins during 30 minutes at 37°C, using a weight/volume ratio (1:20). Undigested tissues of each kind of fish and pepsin were weighed and compared again, without taking into account the weight due to the parasites in the positive samples.

4.2.3. Electrophoretic profile

In addition to the digestions assays, electrophoretic profiles of the two previously selected pepsins were obtained in vertical SDS-PAGE discontinuous gels (10% acrylamide in the separating gel). Electrophoretic separations were carried out at 40 mA/slab, 100V and 150W, using Tris-Tricine buffer (Schäger and von Jagow, 1987) in a Mini Protean® System (BioRad Laboratories, Hercules, USA). Low molecular weight-SDS Marker Kit (GE Healthcare, Buckingham, UK) was employed as reference. The gels were stained with silver**,** following the protocol described by Heukeshoven and Dernick (1985).

4.2.4. New assay conditions

Once the best pepsin formulation was selected after the electrophoretic profile was performed, three modifications were introduced and tested during digestions in fresh and frozen samples, making the fish muscle more accessible to the enzyme action: (1) the use of the selected commercial pepsin, (2) a new weight/volume ratio for digestion solution (1:10 instead of 1:5 that Codex protocol recommends) and (3) the homogenization and flattening of the samples before digestion in a blender for food (Smasher® AES Chemunex). For testing the reproducibility and comparing Codex protocol with the new method after introducing new conditions (hereinafter Liquid Pepsin or LP protocol), a total of 240 digestions were carried out employing at each time 200 g of fresh and frozen hake and mackerel muscles; 120 digestions following the Codex protocol and 120 testing the LP protocol. All assays were carried out with a pepsin concentration of 0.5% at an acidified (pH=1.5 with HCl at 0.063M) pepsin enzyme solution, and incubation temperature of 37°C. After finishing every digestion, undigested muscle residues from each fish type and method were weighed, recorded and compared, without taking into account the weight due to the parasites in the positive samples.

4.2.5. Larvae viability

In order to verify larvae viability during the definitive assays, 40 from the 240 digestions that were carried out were controlled for this aspect (20 digestions of each type of fish species; among them 10 digestions of frozen and another 10 of fresh fishes, and of those 10 digestions, 5 were carried out using each method). Anisakid-positive samples were arranged by introducing 10 larvae of *Anisakis* spp. inside anisakid-negative samples of muscle for digestion. All larvae inoculated were extracted from the muscle where they would be introduced, so larvae inoculating fresh fish samples were alive before digestions (not in the case of frozen fish digestions). Separately, 10 live and free (without muscle) anisakid larvae were digested at 37°C in 1000 ml digestion solution following LP protocol in order to check their integrity after 210 minutes of digestion.

4.3. RESULTS

4.3.1. Samples and pepsin assays

The significance of digestions after using the three different commercial pepsins at the same concentration (0.5%) and different enzymatic activity between them is shown in Table 4.1. This table also illustrates digestion conditions during these assays. The two pepsin formulations that provided higher percentages of digested muscle, both for lean and for fatty fish samples, were 2 and 3.

When both pepsin formulations were compared by equaling their enzymatic activities to 5000 FIP U/g, pepsin 3 showed the least fish residue in both types of fish, as Table 4.2 demonstrates. This table also illustrates commercial pepsins proprieties, their enzymatic activity (in FIP units), the required weight used of each one to equal enzymatic activities, and digestion conditions during these assays.

Table 4.1. Comparison among 3 different commercial pepsins (each one with its own enzymatic activity), in 500 ml of water and 2.5 g of pepsin (at concentration of 0.5%), in an acid solution (pH=1.5) with HCl, at 0.063M. Six assays were carried out using each pepsin, with muscular samples of 25 g of lean (*Merluccius merluccius*) and fatty (*Trachurus trachurus*) fresh fish.

PEPSIN NAME	ENZYMATIC ACTIVITY	FISH MUSCLE (g)	WEIGHT/ VOLUME RATIO	DIGESTION TIME (minutes)	DIGESTION TEMPERATURE (°C)	FISH SPECIES	DIGESTIONS (N)	RESULTING MUSCLE RESIDUE Mean (g) ± SD	DIGESTED MUSCLE Mean (%) ± SD	COMMERCIAL REFERENCE
Pepsin 1	800-2,500 U/mg protein	25	1:20	30	37°C	*Merluccius merluccius*	6	1,048 ± 0.18	95.809 ± 0.7	Sigma Aldrich P7000-100G
						Trachurus trachurus	6	4.933 ± 1.04	80.270 ± 4.15	
Pepsin 2	2000 FIP U/g	25	1:20	30	37°C	*Merluccius merluccius*	6	0,820 ± 0.2	96.719 ± 0.78	Merck 10 1.07190-1000G
						Trachurus trachurus	6	3.180 ± 0.71	87.281 ± 2.83	
Pepsin 3	660U Ph Eur/ml	25	1:20	30	37°C	*Merluccius merluccius*	6	0,085 ± 0.02	99.649 ± 0.12	Panreac (Liquid) 176408.1214-5lt
						Trachurus trachurus	6	1.337 ± 0.45	94.652 ± 1.82	

Table 4.2. Comparison among 2 different commercial pepsins (at different concentration each one; enzymatic activities have been equaled at 5000U FIP), in 500 ml of acid solution (pH=1.5) with HCl at 0.063M. Six assays were carried out using both pepsins, with muscular samples of 25 g of lean fresh fish (*Merluccius merluccius*) and fatty (*Trachurus trachurus*) fresh fish.

PEPSIN NAME	ENZYMATIC ACTIVITY	ENZYMATIC ACTIVITY (FIP)	PEPSIN DOSE (g)	FISH MUSCLE (g)	WEIGHT/ VOLUME RATIO	DIGESTION TIME (minutes)	DIGESTION TEMPERATURE (°C)	FISH SPECIES	DIGESTIONS (N)	RESULTING MUSCLE RESIDUE Mean (g) ± SD	DIGESTED MUSCLE Mean (%) ± SD
Pepsin 2	2000 FIP U/g	2000 FIP U/g	2.5	25	1:20	30	37°C	*Merluccius merluccius*	6	0.881 ± 0.8	96.477 ± 3.2
								Trachurus trachurus	6	1.838 ± 0.9	92.647 ± 3.61
Pepsin 3	660U Ph Eur/ml	543 FIP U/g	9.2	25	1:20	30	37°C	*Merluccius merluccius*	6	0.785 ± 0.19	96.86 ± 0.4
								Trachurus trachurus	6	0.055 ± 0.01	99.78 ± 0.1

4.3.2. Electrophoretic profile

SDS-PAGE profile of pepsin extract 3 showed one band with a molecular weight corresponding to pepsin. However pepsin 2 offered a multiple band profile below to that molecular weight (Figure 4.1), perhaps as autolytic consequence.

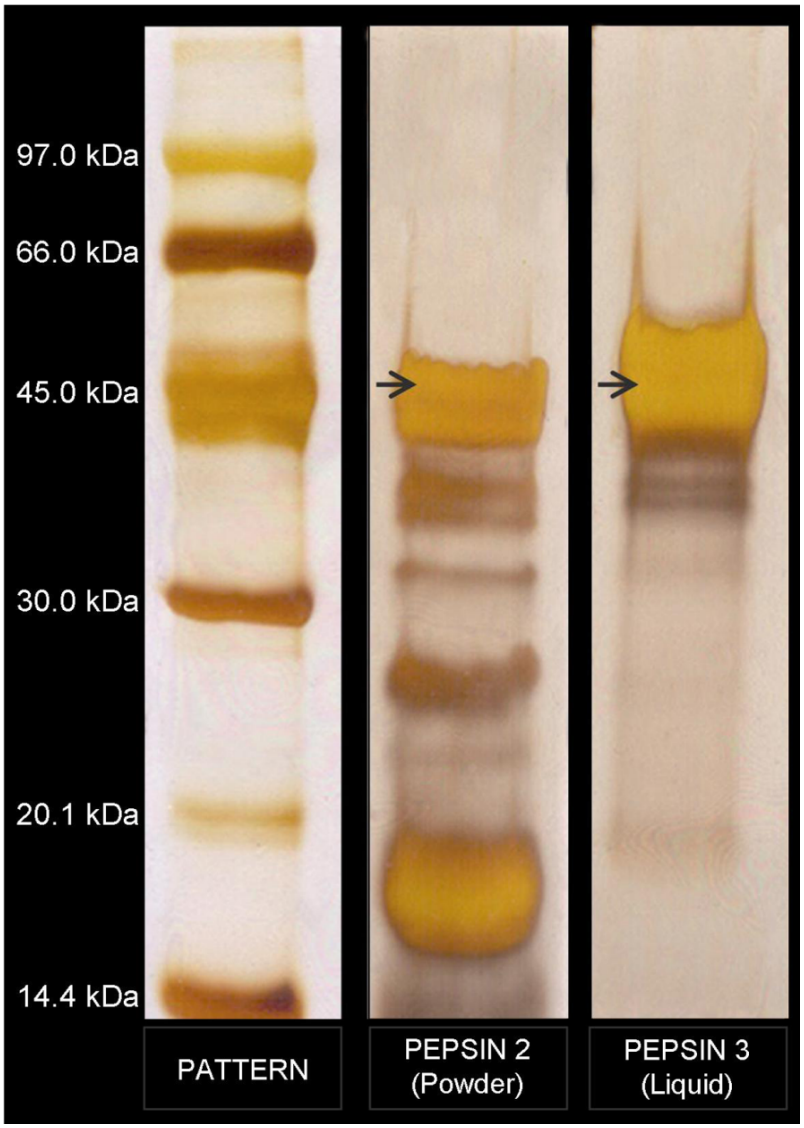

Figure 4.1. SDS-page silver staining profile obtained from the two selected commercial pepsins assayed. Low molecular weight standard (14-97 kDa) from GE Healthcare was used as pattern**.** Additional bands with lower molecular weight than pepsin were obtained at one of them. Black arrow: pepsin band.

4.3.3. New assay conditions

According to obtained results at initial pepsin assays and due to its proteolytic and handling characteristics, liquid pepsin formulation (pepsin 3) was the selected reagent to test the new conditions (LP protocol)

simultaneously to the established and current digestion protocol (Codex). In order to obtain a maximum weight of 1 g of undigested residue in the faster of the two tested methods, for both procedures fresh samples of *M. merluccius* were digested during 20 minutes, and frozen ones for 15 minutes. The reason why 1 g was the determinant weight in order to establish the digestion time with each pepsin and method is because 1 g was the maximum accorded amount of undigested muscle for getting an easy and rapid finding of parasites. Although Atlantic mackerel digestions showed more difficulties during the assays (probably due to muscle characteristics and fat contain), the same criterion of 1 g was followed at the two methods, thus providing more digestion time (45 minutes) to fresh and frozen samples belonging to that species. Results in Table 4.3 show differences in relation to the amounts of undigested muscle residues from lean and fatty fishes and between procedures**.** This table also contains digestion protocols conditions, type of fishes and percentages of digested muscle (%).

New conditions introduced and assayed (liquid pepsin, weight/volume ratio of 1:10 and flattening of the samples before digestion) gave higher percentages of digested muscle (a lower quantity of resulting residue) after less time, both for lean and fatty fish species, than the Codex protocol.

Table 4.3. Resulting muscle residues (g) and digested muscle (%) means, comparing the Liquid Pepsin (LP) protocol (new digestion assay using the selected liquid pepsin -pepsin 3-, at concentration of 0.5% in 2000 ml of water), to CODEX STAN 244-2004 protocol (using the recommended powdered pepsin -pepsin 2-, at concentration of 0.5% in 1000 ml of water). Both digestions were carried out in an acid solution (pH=1.5) with HCl, at 0.063M. A total of 240 assays with samples of 200 g of fish were carried out; 120 for each method (30 assays were performed for fresh, and 30 for frozen lean fish belonging to *Merluccius merluccius*, and the same number for fatty fish (*Trachurus trachurus).*

DIGESTION METHOD	PEPSIN NAME	ENZYMATIC ACTIVITY	FISH MUSCLE (g)	STOMACHER TIME (minutes)	DIGESTION TEMPERATURE (°C)	WEIGHT/ VOLUME RATIO	FISH SPECIES	DIGESTIONS (N)	FISH SAMPLE TYPE	DIGESTION TIME (minutes)	RESULTING MUSCLE RESIDUE Mean (g) ± SD	DIGESTED MUSCLE Mean (%) ± SD
CODEX PROTOCOL (CODEX STAN 244-2004)	Pepsin 2	2000 FIP U/g	200	-	37°C	1:5	*Merluccius merluccius*	30	FRESH	20	125.2 ± 14.91	37.38 ± 7.45
								30	FROZEN	15	126.7 ± 11.22	36.63 ± 5.61
							Trachurus trachurus	30	FRESH	45	32.9 ± 5.33	83.52 ± 2.67
								30	FROZEN	45	36.48 ± 4.61	81.76 ± 2.3
LP (LIQUID PEPSIN) PROTOCOL	Pepsin 3	660U Ph Eur/ml	200	4	37°C	1:10	*Merluccius merluccius*	30	FRESH	20	0.653 ± 0.328	99.67 ± 0.16
								30	FROZEN	15	0.475 ± 0.184	99.76 ± 0.09
							Trachurus trachurus	30	FRESH	45	0.902 ± 0.24	99.55 ± 0.12
								30	FROZEN	45	0.795 ± 0.18	99.60 ± 0.09

4.3.4. Larvae viability

Concerning larvae viability tests, after both Codex and LP digestion protocols for both type of fishes and for both forms of preservation, all larvae introduced were recovered in perfect conditions; live larvae were recovered still alive and showing a good mobility, resembling to mobility showed before digestions (Figure 4.2). Moreover, the 10 live and free larvae which were submitted to 210 minutes of digestion following the LP protocol, were recovered without mobility but completely entire.

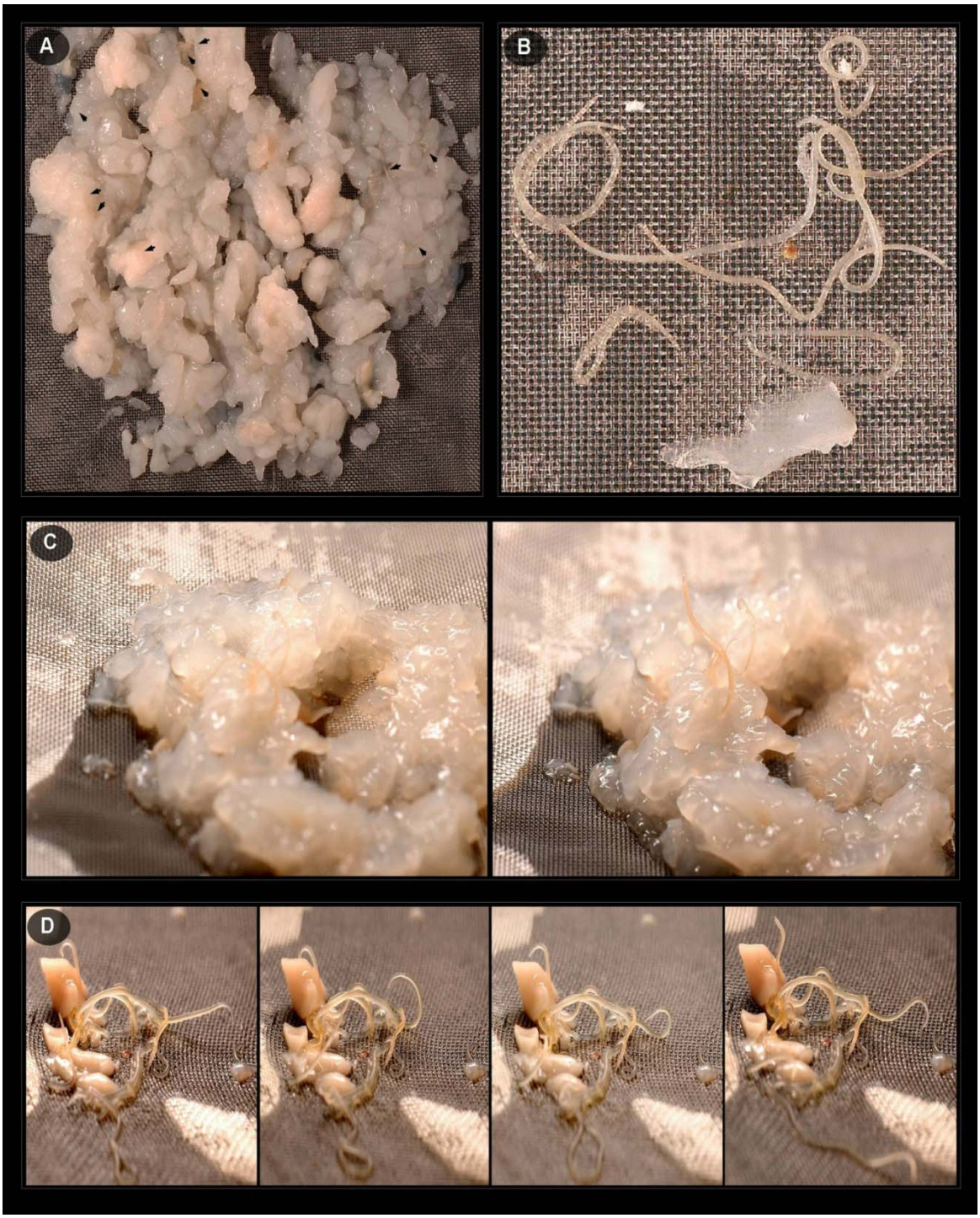

Figure 4.2. (A-D). Resulting digestions after examining and controlling the viability of the larvae. **A:** Ten anisakid larvae after Codex digestion protocol of frozen *Merluccius merluccius.* Black arrowhead: anisakid larval. **B:** Ten anisakid larvae after LP (Liquid Pepsin) digestion protocol of frozen *Merluccius merluccius.* **C:** Sequence of two pictures showing live anisakid larvae moving after Codex digestion protocol of fresh *Merluccius merluccius.* **D:** Sequence of four pictures showing live anisakid larvae moving after LP digestion protocol of fresh *Merluccius merluccius*.

4.4. DISCUSSION

Due to the low confidence of other traditional parasite detection methods like the widely used visual inspection of abdominal cavity (Llarena-Reino et al., 2012), the norm by Codex (CODEX STAN 244-2004) is considered the current recommended procedure for anisakids detection and counting in certain fish species and commercial displays. However, due to the lack of an officially legislated reference standard, not for Codex protocol neither for any of the traditionally used formulas, there is no consensus in *modus operandi* to accomplish with artificial digestions for anisakids detection. An example of a similar approach in terms of performance and objectives, which has been sharply and effectively legislated, is the detection method for trichinellosis. Traditionally different detection protocols and variations had been used for meat inspections and for studies concerning *Trichinella* (Forbes and Gajadhar, 1999; Leclair et al., 2003; Gajadhar et al., 1996 and 2009). Since January 2006, a Commission Regulation of the European Community of 5 December 2005 ((EC) 2075/2005) has laid down specific rules on official controls for *Trichinella* in pig meat. This detailed law required laboratories to carry out the magnetic stirrer protocol for pooled-sample digestion in fresh pig meat. Afterward, some authors concluded that pepsin powder formulations potentially caused severe allergic reactions to sensitive people (Marqués et al., 2006) and workers (Maddox-Hyttel et al., 2007) who handled the chemical, thus constituting a health risk. Simultaneously, the Commission Regulation of the European Community of 24th October 2007 ((EC) 1245/2007) modified Annex I of the regulation (EC) 2075/2005, allowing the use of liquid pepsin formulations to detect *Trichinella* in meat. Similarly, during the present study the artificial digestion protocol from Codex has been revised in depth, detecting some limitations and disadvantages in powder pepsin forms and in the conditions. During the first assay carried out with the three pepsin formulations (digestions at the same concentration of 0.5%; Table 4.1), pepsin 1 offered the lowest proteolytic effectiveness. Due to this, it was removed from the study. After selected and assayed pepsin formulas 2 and 3 for the second test (digestion solutions with the same proteolytic activity; Table 4.2), pepsin 3 gave better results; higher percentage of digested muscle than pepsin 2. Therefore, liquid pepsin form (pepsin 3) was more effective, and it also offered an easier handling at work procedures than pepsin formulation 2. Moreover and as mentioned above, liquid enzyme formula avoids possible allergic reactions that pepsin in powder form may cause. Additionally, the study of the purity by means of the SDS-PAGE silver staining profile was determinant to qualify pepsin formulation 3 as the cleanest, purest, fastest and the most versatile and efficient of both. This was the reason why this liquid enzyme form was selected as the most interesting pepsin to be assayed applying the settings and new conditions suggested in

this study, in a comparative test between both pepsin formulations and both procedures (Codex and LP). That comparison revealed that LP protocol is more sensitive, efficient and accurate. It offers innovative characteristics like being more handy and easier to use than Codex, even for unskilled personnel such as fish markets and factories workers. Therefore, since there is a non-standardized safer optional method for *Trichinella* detection, it seems reasonable to consider a similar non standardized safer alternative method for anisakids detection as well, due to the important, increasing and urgent requirement of its use.

Besides increasing safety, comfort and usability, this novel procedure reduces costs and test times. This fact leads to a huge reduction of the expenses and time dedicated to quality and public health controls at industries, without variation on results reliability. These kinds of improvements are extremely significant, also for research centers to make faster progresses in specific aspects of the parasites and the public health prevention programs.

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CHAPTER 5

Diagnostic Methods (III)

New advances in imaging detection methods

UV-light and confocal studies: new advances in imaging detection methods for the presence of anisakids in the flesh of commercial fish species, with a view to an industrial application

ABSTRACT

The third-stage larvae of parasitic nematodes of the family Anisakidae are present in the visceral cavity and surrounding areas of many stocks of commercial marine fish species from the North Atlantic, occurring at very high prevalence. Human anisakiasis and hypersensitivity reactions are the most important consequences after the consumption of raw or undercooked fish products containing anisakids, especially *Anisakis simplex,* the most commonly parasite associated with these human diseases. Fresh specimens from the horse mackerel *Trachurus trachurus*, anglerfish *Lophius* spp. and European hake *Merluccius merluccius* acquired at retail in Vigo (Spain), were processed by the press method and examined under UV illumination by using an UV-Cabinet as a high-throughput screening tool, for the presence of anisakid larvae in muscle. UV-light examination of fish specimens deepened in the knowledge of this technique to improve its use in the fish processing industry. The absorption and emission properties of parasites and the specific principles that make these biological hazards having auto-fluorescence could be revealed by first time by confocal analysis. These findings could increase the knowledge about this problem and have stressed the importance of implementing more prophylactic measures at the consumer level, as well as the need of monitoring programmes for preventing unhealthy and anaesthetic parasites in the flesh of commercial fish species. Therefore, the development of faster, more efficient and affordable anisakids detection procedures for fresh and frozen fish lots will contribute to reduce the level of rejections or complaints from customers, thus guaranteeing a better global advertisement of these products.

KEYWORDS

Anisakids; auto-fluorescence; confocal; fish; UV

5.1. INTRODUCTION

Anisakis simplex and *Pseudoterranova decipiens* are the species of nematodes more frequently present in many commercial fish species (Marty, 2008; Jurado-Palomo et al., 2010), and most commonly associated with human zoonoses. Although most of the larvae are usually found in the abdominal cavity of fish, the parasitic burden present in the flesh is sufficient to affect food safety. The consumption of fish products containing live L3 larvae can result in anisakiasis (Van Thiel et al., 1960; Butt et al., 2004), while hypersensitivity reactions to parasite antigen may occur after eating fresh, previously frozen, or cooked fish products (Kasuya et al., 1990; Audicana et al., 1995, 2002; Werner et al., 2011). Moreover, the presence of nematodes is having a significant impact on fish consumption since quality of commercial species is seriously being affected (Fischler, 2002). Consumers increasingly reject seafood products due to

an improved awareness about marine parasites, and because of a higher prevalence of this biological hazard when compared with several years ago.

According to EU regulation (EC) 853/2004 and Commission Regulation (EC) 2074/2005; seafood companies must ensure that fishery products intended for commercial activity have been visually inspected for parasites during trimming and after filleting. Where candling of fillets is necessary, it must be included in the sampling plan of processing plants. It must be carried out on a light table holding up fish to a light in a darkened room (Figure 5.1).

Figure 5.1. Candling procedure. Fillets of *Scomber scombrus* examined on a light table. Black arrow heads: anisakid larvae within the fish muscle.

Any seafood product obviously contaminated with parasites must not be placed on the market. However, as some authors have recently stated, skeletal muscle of fishes is not routinely examined as part of the EU legislative programs, and in the best case an undetermined representative number of individuals are inspected by making indirect observations of viscera and gut cavity (Marty, 2008). Scientific studies have related anisakids in the viscera and muscle confirming the low efficiency of visual inspection of gut parasites as a commonly recommended method for predicting nematode larvae in the flesh of fish (Llarena-Reino et al., 2012). In addition, visual inspection and candling of fillets are not suitable for quantitative determination of parasites in fish. Even though this kind of “non-destructive” scheme of inspection does not guarantee the commercialization of parasite-free fish because parasite burdens are being underdetected (Levsen et al., 2005), no other method as a golden standardization has been

accepted as the international reference protocol accomplish with industrial requirements (Llarena-Reino et al., 2013). Precision of prevalence estimates of anisakids in skeletal muscle of fish lots should be enhanced by using quantitative and more sensitive, fast, efficient and manageable diagnostic methods such as enzymatic digestion (Llarena-Reino et al., 2013) or UV illumination. Concerning this last imaging procedure, in 2010 Levsen and Lunestad carried out an approach in order to help work speed, utilizing the auto-fluorescence of frozen nematodes previously described by Pippy (1970) and Karl and Leinemann (1993). The method, based on visual inspection of flattened/pressed and deep-frozen fish fillets or viscera under UV-light, allowed the processing of a larger sample number per time unit. However, to date no one has described the fluorescent emission pattern and the basic principles of auto-fluorescence of anisakids larvae. On the basis of this, advances in parasitic detection methods to be included in self-control programs are a key issue within the fishing sector.

The capacity of allowing the visualisation of biological samples with a much higher resolution and sensitivity, creating 3D images, and following specific cellular reactions over periods of time, have converted confocal microscopy into a much more sophisticated imaging instrument than conventional light microscopy (Inoué, 2006). This technology is based on a small spot, usually derived from a focused laser beam, which illuminates an object. The target point can be observed with a spatially restricted optical system so that only signals emanating from this spot are detected (White et al., 1987). The possibility of carrying out analysis at specific wavelengths of light, and the rejection of interfering signals from out-of-focus structures, which often has seriously degraded images, also reinforces the use of this instrument as a useful tool for biological research (Amos et al., 1987; White et al., 1987; Paddock, 2000). This technology, firstly patented and later re-described by Minsky (1957; 1988), did not elicit a great deal of interest since the late 1980's, when lasers, computers and digital technology were introduced and integrated. In the field of cell and molecular biology, it has been a major breakthrough by giving a great versatility in fluorescence imaging (Hibbs, 2004).

The purpose of this chapter is to determine the fluorescent emission pattern and the basis of the auto-fluorescence of *Anisakis simplex* larvae, for the future development of an easy to use and affordable imaging tool to industrially detect anisakids present in the edible part of commercial fish lots. The needing of improved and faster diagnostic methods for fish inspection to be included in self-control procedures at seafood companies has become a priority, since fish quality and safety are being seriously affected by the presence of parasites.

5.2. MATERIALS AND METHODS

5.2.1. Ultraviolet fluorescence

Fresh fish individuals belonging to the three species, horse mackerel *Trachurus trachurus*, anglerfish *Lophius* spp. and European hake *Merluccius merluccius* obtained in retail fish establishments at Vigo (Spain), were gutted, manually skinned, and thinly-sectioned (maximum 10 mm thick) in order to apply a press method based on the technique described by Pippy (1970), Karl and Leinemann (1993) and Levsen and Lunestad (2010). To this end, sliced left and right-side fillets (incl. belly flaps) were placed in transparent resealable plastic bags and then compressed to 2 mm thickness by using a hydraulic press Mega 30 Ton KMG-30 (Melchor Gabilondo, S.A., Spain) (Figure 5.2.A). After further frozen at -20°C for a minimum 12 hours, pressed fillets were visually inspected under an UV-light source in dark conditions, using a Vilbert Lourmat CN-15LC Cabinet (Vilbert Lourmat, Marne La Vallée, France) (Figure 5.2.B) at 300-400 nm wavelength measure range, 365 nm peak excitation and 1300 µW/cm^2 peak irradiance. In order to take full advantage of the auto-fluorescence of dead anisakids, several images were captured with a camera Nikon D200 with lens AF-S Micro Nikkor 60 mm f/2.8G ED (Nikon Corporation, Tokyo-Japan) employing complete samples as measure area. Then, the picture of each entire sample was viewed for parasite counting and any kind of artefact present within the bags, were distinguished from parasites. Any part of the image, even shape and size, was enlarged for finer fluorescence resolution for parasite confirmation. In cases of any doubt on parasite counting, the pepsin-HCl digestion method was used as a confirmatory golden method following Llarena-Reino et al. (2013).

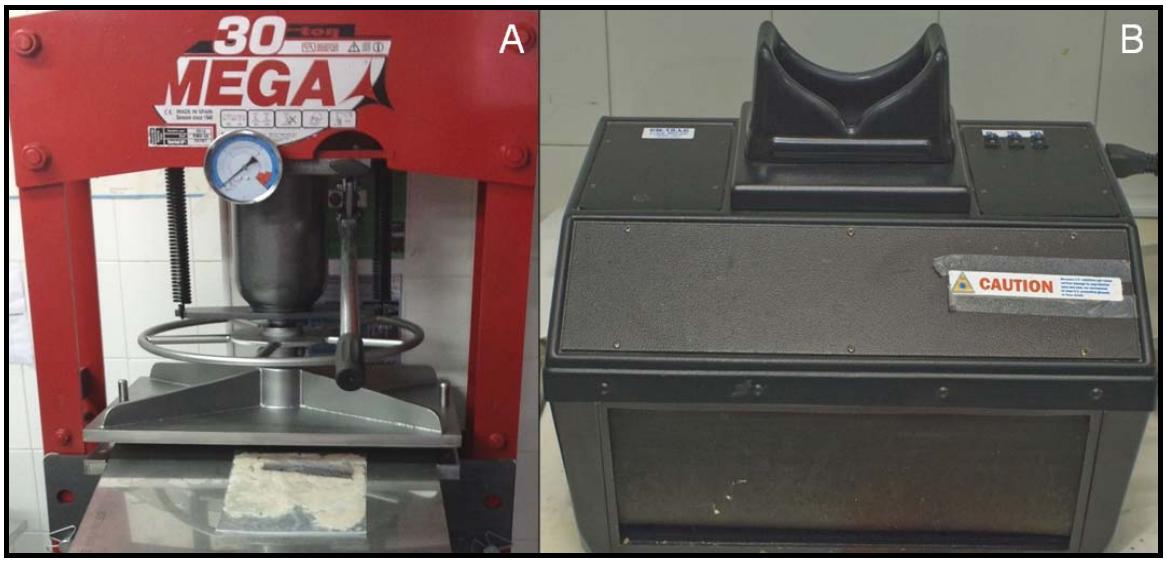

Figure 5.2. (A-B). A: Hydraulic press Mega 30 Ton KMG-30 utilized to press filleted fishes contained in transparent resealable plastic bags. **B:** Image of the Vilbert Lourmat CN-15LC cabinet that was subsequently used to visualize the pressed samples under UV-light.

Specific data about the environmental conditions, as temperature and relative humidity, were provided by a Thermohygrometer Datalogger Testo 0563 1775 177-H1 (Instrumentos Testo, S.A. Cabrils, Barcelona-Spain), during the test.

5.2.2. Confocal analysis

Confocal imaging analysis was conducted in the Confocal Microscopy Unit of the Biological Research Center (CIB-CSIC) in Madrid (Spain). The study was carried out by means of a laser scanning spectral confocal microscope Leica TCS SP2 equipped with AOBS system (Leica Microsistemas S.L.U., Barcelona-Spain) (Figure 5.3).

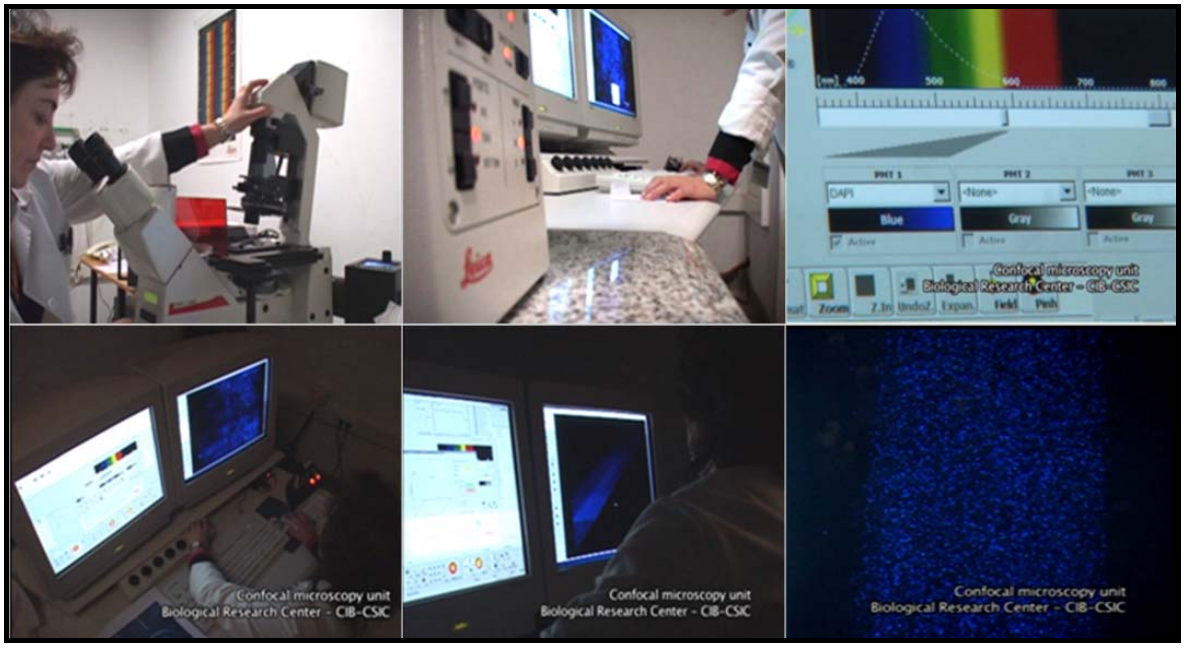

Figure 5.3. Confocal microscopy unit of the Biological Research Center (CIB-CSIC), Madrid (Spain) during imaging studies carried out with the laser scanning spectral confocal microscope Leica TCS SP2.

A sample panel of nematode larvae of *Anisakis simplex* collected from the horse mackerel *Trachurus trachurus*, anglerfish *Lophius* spp. and European hake *Merluccius merluccius*, previously preserved at -20°C for a minimum 12 hours, was provided by the central node of the PARASITE-Biobank (IIM-CSIC) sited in Vigo (Spain). To determine the emission wavelength pattern of *Anisakis simplex,* the scanning of a finely focussed UV-laser spot across each parasite was completed at a wavelength of 365 nm as spectral source (power 20 mW). Samples were UV irradiated from the upper vertical up to 250 mm distance, and a camera Basler Scout situated at 300 mm distance and 45° from the sample was used to capture images. Several regions of interest (ROIs) within the samples were selected with the aim of obtaining information about the fluorescence emission pattern in different spots of each larval, thereby achieving a more detailed description of the nematodes absorption and emission properties. Moreover, the application of five different shock treatments to kill anisakids was studied by means of the confocal imaging technique with the aim of evaluating and comparing their emission spectra. Cryostat, paraffin, formalin, microwave and liquid nitrogen were the treatments applied on selected samples. The higher or lower potential to

break external parasitic cuticle for allowing the visibility of fluorochromes, was analyzed by selecting ROIs on parasites after the application of each shock proceeding.

Temperature and relative humidity during the confocal imaging test were also provided by a Thermohygrometer Datalogger Testo 0563 1775 177-H1 (Instrumentos Testo, S.A. Cabrils, Barcelona-Spain).

5.3. RESULTS

5.3.1. Ultraviolet fluorescence

Complete pressed samples were inspected and photographed in the UV-Cabinet as individual images for parasite counting. After deleting distinguishable artefacts as fish bones, spines, and small fragments of nerve tissue, remnants of skin or tissue lining in the fillets, nematodes were observed showing a bright bluish-white appearance, and were easily detected as fluorescent white spots, nodules or patches against a darker background of the pressed muscle tissue (Figure 5.4). Sometimes, very few anisakid larvae were missed due to the weak or discontinuous fluorescence of melanised black capsules (Figure 5.5). Detection efficiency was 100% by comparing parasite counts by the press method and parasite recoveries in re-examined samples after artificial digestion in contaminated fish flesh from each fish species.

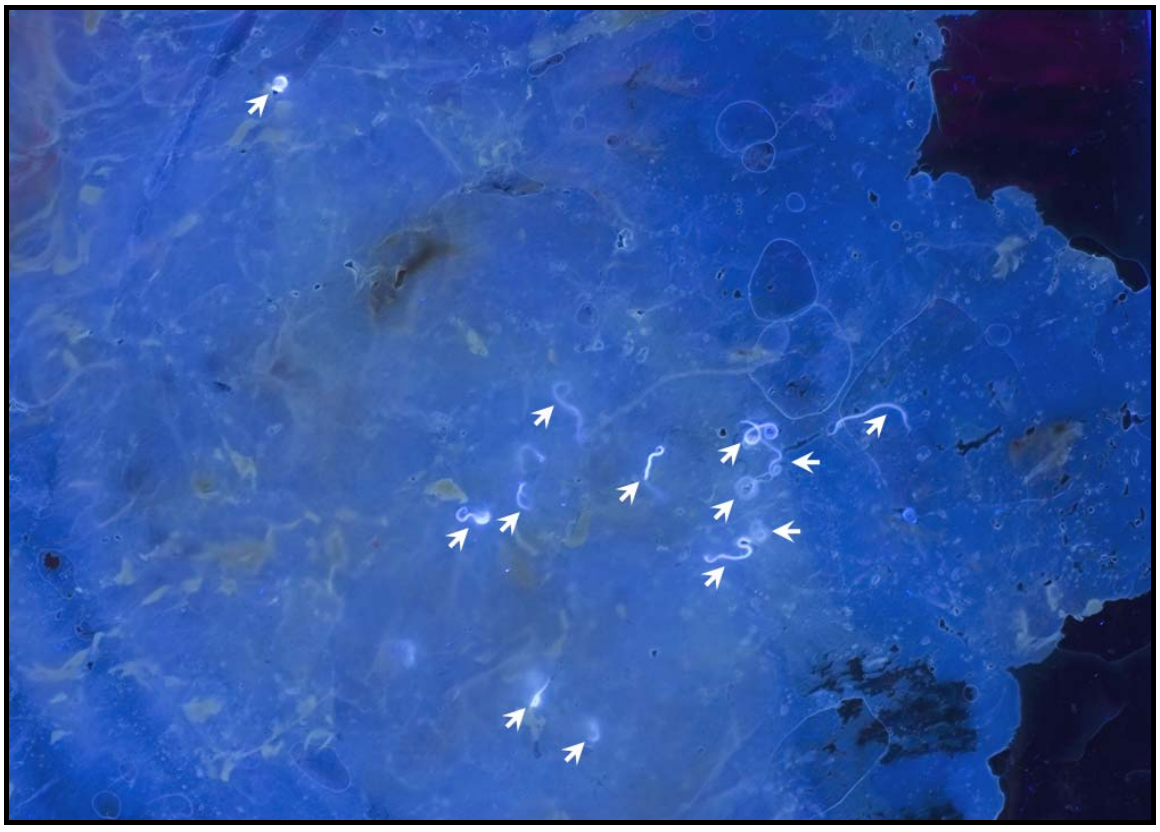

Figure 5.4. Image of a pressed fillet of *Merluccius merluccius* observed under UV-light in a Vilbert Lourmat CN-15.LC cabinet. Highly contrasting bluish-white spots (arrow heads) represent anisakid larvae within the fish muscle.

Temperature and relative humidity during the test were 24.1°C and 57.2% respectively.

Figure 5.5. Detail of an image of a pressed fillet of *Merluccius merluccius* under UV-illumination inside a Vilbert Lourmat CN-15.LC cabinet, which shows areas of weak and discontinuous fluorescence due to the existence of melanised black capsules embedded in the flesh.

5.3.2. Confocal analysis

Lambda scan records were obtained for several larvae previously extracted from the hosts. Resulting fluorescent images were detected at a specific emission wavelength within a user-defined wavelength range. The confocal analysis confirmed the intestinal region as the location where lipofucsin granules reside. Lambda scan was then used to measure the emission spectrum of fluorochromes, and provided specific absorption properties of the nematodes measured in image series by using intestinal ROIs (Figure 5.6 and Figure 5.7). Lambda scan analysis of ROIs within the samples confirmed the same emission auto-fluorescence pattern of selected spots in the intestine of a given anisakid, even though each ROI emitted at a different mean intensity value (Figure 5.8). Nematode auto-fluorescence was localized in discrete 1-5 µm granules (Figure 5.9) dispersed throughout the intestine and yielded absorbance in the UV range with peaks of maximum emission corresponding to 430-440 nm. The above refining confocal analysis allowed us to discriminate errors or artefacts in the fillets which also fluoresced and occasionally affected reading.

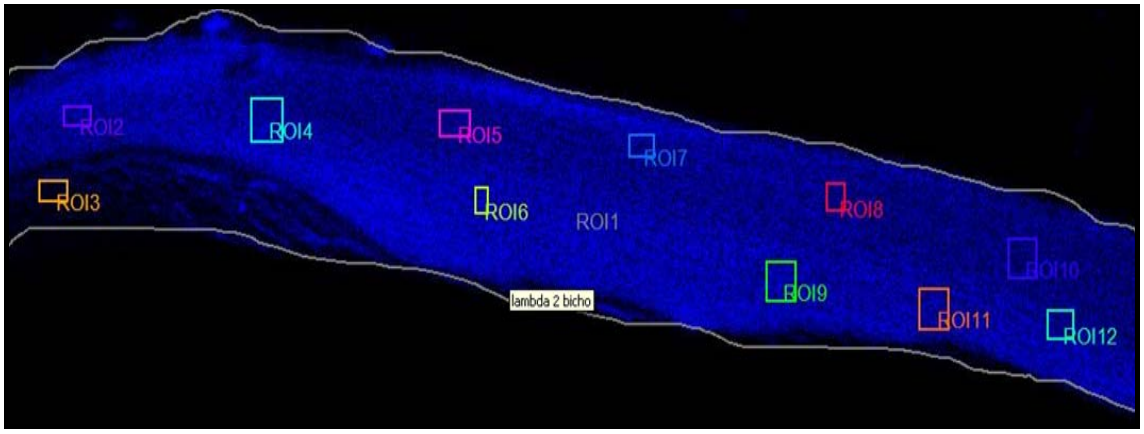

Figure 5.6. Image extracted from laser scanning spectral confocal microscope. The intestinal area of an anisakid after applying an excitation source of 365 nm wavelength is illustrated. Coloured squares highlight the twelve ROIs selected for this specific sample. Each ROI includes a high number of lipofucsin granules.

Spectrum

	ROI1	ROI2	ROI3	ROI4	ROI5	ROI6	ROI7	ROI8	ROI9	ROI10	ROI11	ROI12
Mean Value	38.67	45.97	10.86	45.14	38.65	54.29	42.43	37.74	48.42	33.23	46.04	40.54
Pixel Count	7661200	18000	18750	44800	27000	11000	17600	16000	37700	36250	40300	25300
Pixel Sum	296.29 10E6	827544	203707	2.02 10E6	1.04 10E6	597162	746700	603917	1.83 10E6	1.2 10E6	1.86 10E6	1.03 10E6
Length	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm	225.00 nm
Frame Count	50	50	50	50	50	50	50	50	50	50	50	50
Variance	2082.77	2724.62	180	2698.65	2041.52	3928.04	2463.01	2075.27	3201.37	1686.84	3015.48	2411.54
Standard Deviation	45.64	52.2	13.42	51.95	45.18	62.67	49.63	45.56	56.58	41.07	54.91	49.11
Average Deviation	39.41	45.65	11.35	45.04	39.3	54.73	42.72	39.32	48.75	35.11	47.29	42.29
Max Amplitude	128.26	145.38	39.54	144.04	127.95	178.24	138.6	127.65	163.98	117.17	155.9	141.33
Max Position	439.29 nm	439.29 nm	430.10 nm	439.29 nm	439.29 nm	439.29 nm	434.69 nm	434.69 nm	439.29 nm	434.69 nm	434.69 nm	434.69 nm
Min Amplitude	730.96 10E-6	0	0	0	0	0	0	0	0	0	0	0
Min Position	402.55 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm	375.00 nm
Center Of Mass Pos.	460.07 nm	462.57 nm	458.55 nm	461.47 nm	460.94 nm	461.04 nm	460.55 nm	458.88 nm	460.48 nm	457.79 nm	459.29 nm	458.57 nm

Figure 5.7. Set of confocal imaging parameters resulting from the spectrum of the intestinal ROIs selected in one of the anisakid samples analyzed.

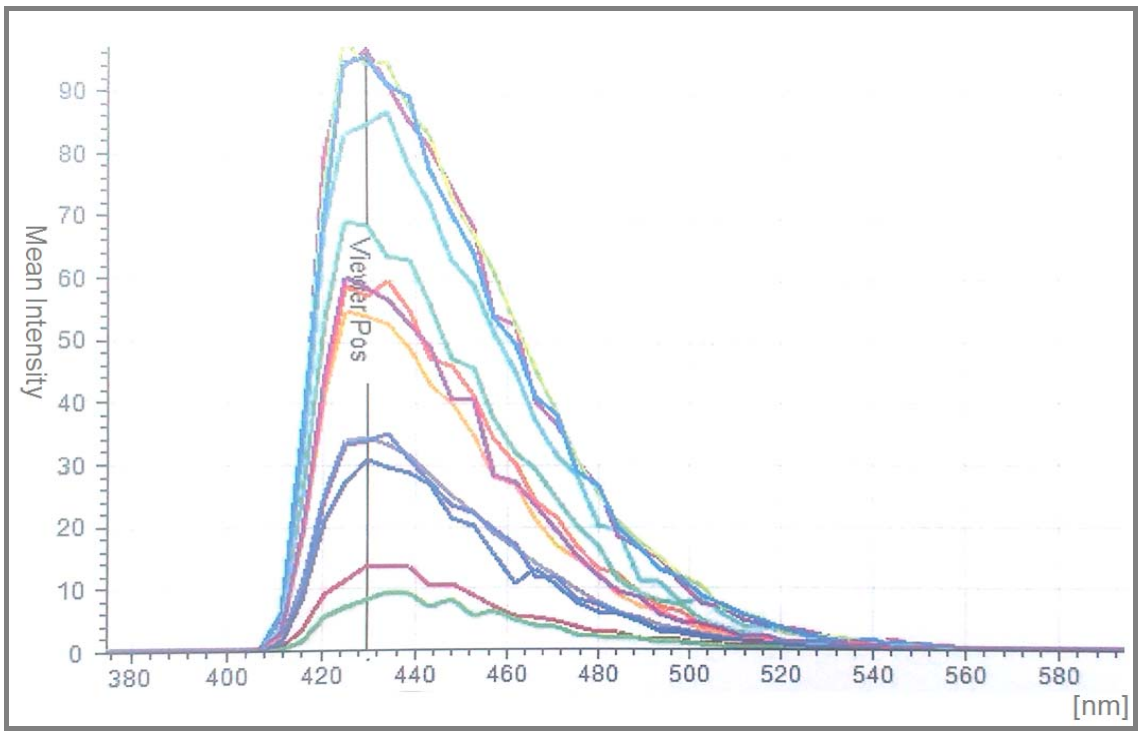

Figure 5.8. Lambda scan analysis of an anisakid larval extracted from a fish specimen preserved at frozen conditions. Coloured lines following the same emission auto-fluorescence pattern represent the ten ROIs selected for this specific sample.

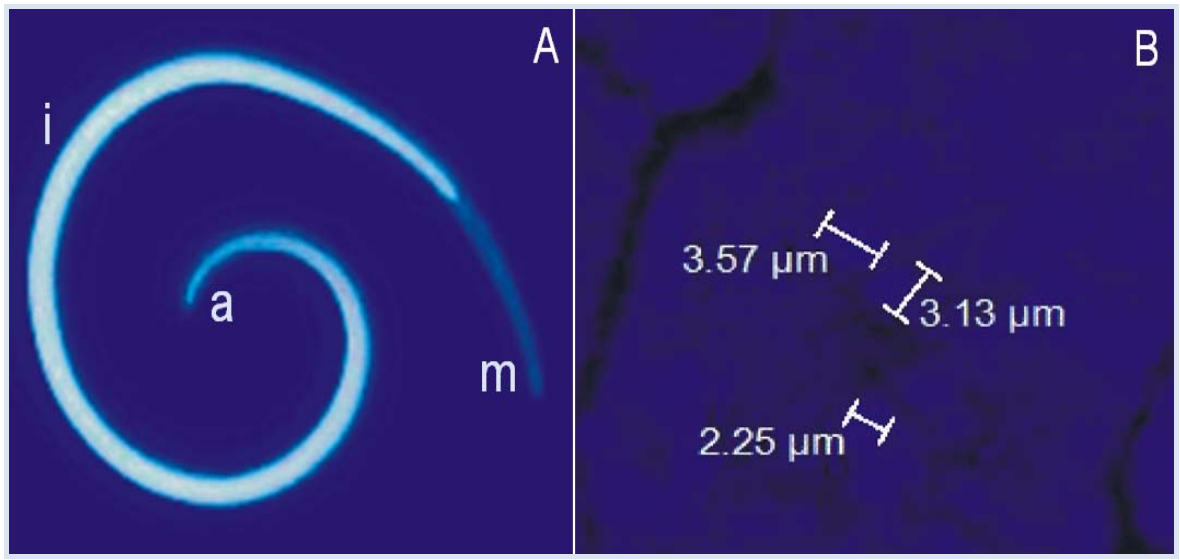

Figure 5.9. A: Image extracted from the UV-cabinet showing a nematode inside a pressed frozen fillet of fish emitting auto-fluorescence, especially along the intestinal region (m: mouth, i: intestine, a: anus). **B**: Detail of a confocal image illustrating 1-5 µm granules of lipofucsin located in the intestine of nematodes.

In relation to the comparison carried out among shock treatments on parasites, lambda scan analysis showed mean intensity rates evidencing high differences among ROIs and among samples, depending on the procedure chosen in every particular case (Figure 5.10). Especially after formalin treatment, mean intensity values were unexpectedly low (less than 50 Mean Fluorescence Intensity, MFI (AU)) for almost the total number of ROIs within the four parasites processed. In addition, maximum wavelengths emitted by parasites showed values exceeding the estimated range in the use of formalin at one of the four samples, and also in the case of paraffin. For those cases, the emission auto-fluorescence pattern was characterized by presenting evident disparities among ROIs, and differences when comparing to samples without any shock treatment after the frozen process of hosts. However, resulting confocal data after applying liquid nitrogen and cryostat, revealed a similar emission auto-fluorescence pattern than frozen samples. Finally, the use of microwave gave the best results concerning mean intensity values and wavelength rates; all samples appeared showing peaks of maximum wavelength emission of 425-444 nm, and following the same emission auto-fluorescence pattern than frozen, liquid nitrogen and cryostat samples (Table 5.1). After quantitative comparative confocal imaging study by analyzing lambda scan records, striking differences were visually appreciated among treatments by observing the fluorescent images. The density and the intensity of brightness emitted by lipofucsin granules were qualitatively consistent with numerical results previously exposed (Figure 5.11).

During confocal imaging tests, environmental conditions (temperature and relative humidity) were 23.8°C and 55.7% respectively.

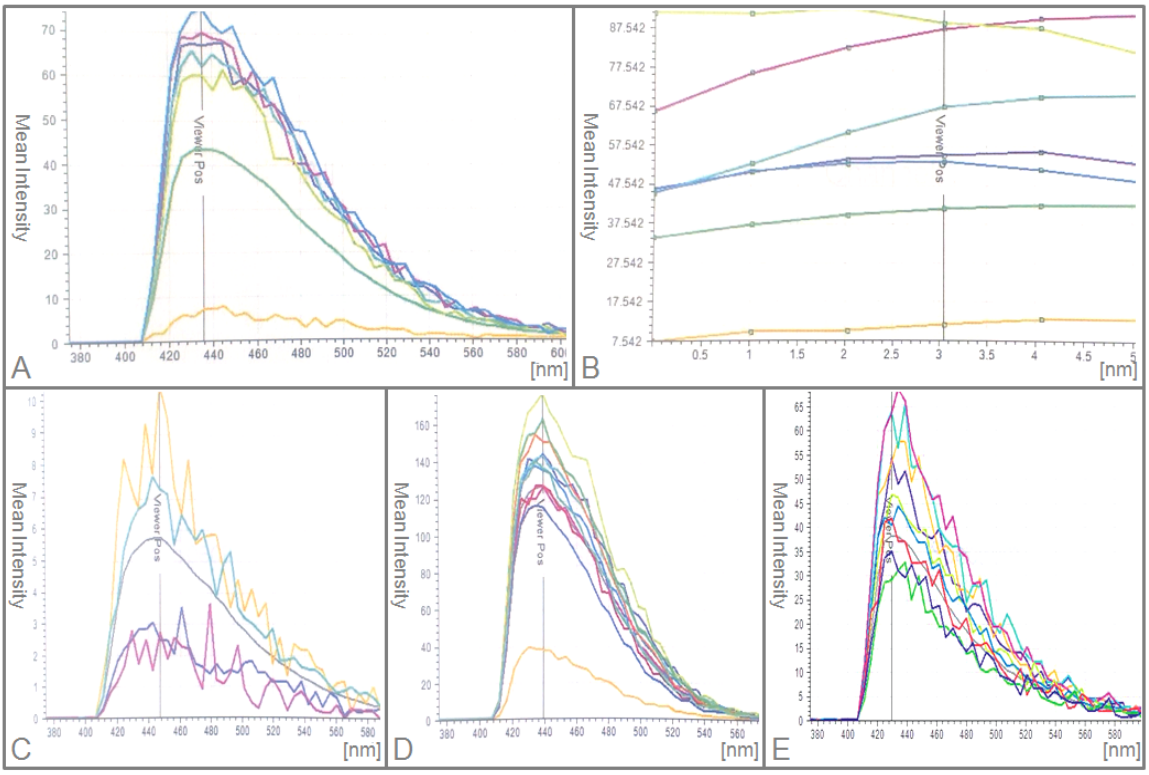

Figure 5.10. (A-E). Lambda scan records of five different anisakid larvae after treatment with five shock treatments. Coloured lines represent selected ROIs for each sample emitting auto-fluorescence under an UV-light excitation source of 365 nm wavelength. **A**: Cryostat. **B**: Paraffin. **C**: Formalin. **D**: Microwave. **E**: Liquid nitrogen.

Table 5.1. Confocal tests carried out on anisakid larvae treated with five shock treatments. The number of ROIs analyzed, MFI (AU) ranges, and auto-fluorescence emission wavelength ranges, λ (nm), for each sample studied are given.

Treatment	Confocal Test 1	Confocal Test 2	Confocal Test 3	Confocal Test 4
Cryostat	ROIs: 7 MFI (AU): 7.92-75.51 λ (nm): 431.08-445.1			
Paraffin	ROIs: 7 MFI (AU): 13.33-93.5 λ (nm): 2.04-5.09			
Liquid nitrogen	ROIs: 10 MFI (AU): 32.63-68.84 λ (nm): 429.86-439			
Microwave	ROIs: 9 MFI (AU): 41.8-95.29 λ (nm): 430.1-439.29	ROIs: 10 MFI (AU): 18.56-84.38 λ (nm): 425.29-443.57	ROIs: 12 MFI (AU): 39.54-178.24 λ (nm): 430.1-439.29	ROIs: 13 MFI (AU): 9.25-98.9 λ (nm): 425.29-434.43
Formalin	ROIs: 5 MFI (AU): 3.56-10.55 λ (nm): 442.96-479.2	ROIs: 8 MFI (AU): 20.59-58.6 λ (nm): 435.82-452.22	ROIs: 8 MFI (AU): 0.95-120.84 λ (nm): 433.9-597	ROIs: 9 MFI (AU): 1.43-93.42 λ (nm): 424.84-438.43

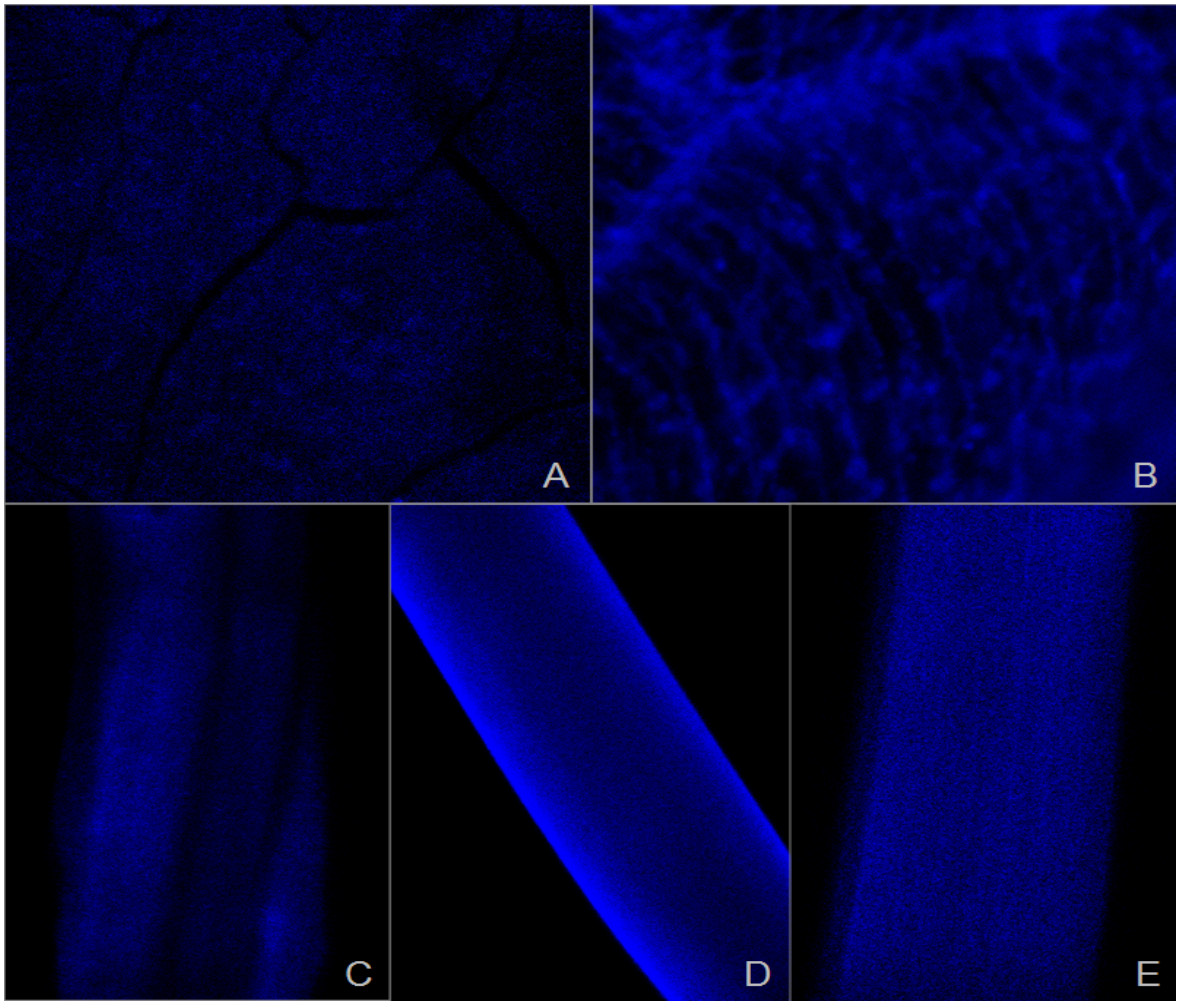

Figure 5.11. (A-E). Confocal images of anisakid larvae observed under 365 nm wavelength of UV excitation, after applying five different shock treatments. A high number of lipofucsin granules can be distinguished emitting auto-fluorescence. **A**: Cryostat, 63X. **B**: Paraffin, 63X. **C**: Formalin, 20X. **D**: Microwave, 20X. **E**: Liquid nitrogen, 20X.

5.4. DISCUSSION

The use of qualitative muscular inspection procedures (e.g. candling, gross visual inspection) in fish processing during self-controls, as international regulations recommend, may significantly decrease the recorded values of muscular parasites found, mostly due to the low detection efficiency of these non-destructive methods. However, these measures, designed to minimize potential health risks related to the presence of anisakid L3 larvae in seafood products, do not prevent from allergenic reactions in consumers and occupational asthma in fish-farming workers (Nieuwenhuizen et al., 2006), nor protect companies from aesthetical impact and loss of quality in the products offered for sale. Levsen et al. revealed in 2005 that only 7 to 10% of nematode larvae present in fish fillets were detected by candling. A few years later, Celano et al. (2013) confirmed a lower effectiveness of visual inspection compared to the UV

transillumination method, thus stating that the official method used in the European Union for anisakid detection in fish samples does not provide a sufficient guarantee of muscular larvae recovery.

With regard to the auto-fluorescence observed in anisakids in the present work, it is well known that nematodes accumulate a fluorescent compound used as an indicator of its viability; lipofuscin (Forge and MacGuidwin, 1989), which becomes less sensitive to ultra-violet radiation as parasite ages (Davis et al., 1982; Klass, 1977). The fluorescence has been attributed to hydrolysis of fluorescein diacetate by esterases, which is dispersed throughout the intestinal region upon nematode death. Auto-fluorescent granules herein observed accumulating in the intestinal tract of anisakids match those previously described in many other nematodes, in size, distribution and colour. Regardless of the parasite species, the auto-fluorescence was dispersed in 100% of frozen anisakids analyzed. This ensures the reliability of the press method employed, which showed a 100% detection score in parasite counting as confirmed by the artificial digestion method.

The application of UV transillumination for detecting muscular anisakid larvae has shown a high detection score and allowed the examination of a comparatively large amount of samples in a reasonable short period of time. In the frozen fish processing industry, it can be an interesting alternative to expensive and time-consuming methods in use, as molecular techniques, especially when many samples require a quantitative inspection. As example of this, spectral automatic nematode detection is a prioritized research line for the cod fillet industry, where several methods have been tested; some at laboratorial scale (Sivertsen et al., 2011; Heia et al., 2007) and one under industrial conditions (Sivertsen et al., 2012). The knowledge acquired on the basis of confocal results here obtained, makes it possible to advance, in the field of fish processing industry, in the progress of efficiency at invasive imaging inspection methods. Confocal has been demonstrated to be a promising technology able to contribute very positively to provide substantial improvements in visibility and resolution of auto-fluorescent samples, through a continuous progress of quantitative and more reliable practices as UV-technique.

In addition, determined shock treatments susceptible to be used for killing anisakids are more advantageous than others to be incorporated in routine imaging detection programs of parasites, due to their effectiveness in breaking parasitic cuticle and allowing the visibility of lipofucsin granules under an UV-light source. Despite the non-availability of commercial specific equipments, and although implementation of UV-inspection at fish processing plants should be previously adjusted to the needs of each particular company, liquid nitrogen, formalin and particularly microwave are the best techniques to use before UV-inspection. Although many of these treatments have not been tested in standardized conditions, progress beyond the SoA have to be carried out with the aim of ascertain how the different anisakid species respond to the each different treatments, and the role of habitat, host and storage conditions. Consequently, they should be taken into account with a view to its inclusion and application on monitoring programs to prevent the commercialization of parasitized fish lots. In accordance with this

approach, in the future, computer-image schemes must be designed for images processing, thus allowing a more clearly viewing at once, and quick parasite counting on all images of each entire sample. The need for a repeatable and automatic counting system than the visual counting on the UV-Cabinet is essential for industrial implementation. Such improvements could make UV-inspection a user-friendly assessment tool within a HACCP programme during on-site fish inspections. Its speed, easy handling, efficiency, the possibility of knowing number and anatomical location of parasites in the fillets, and the chance to make possible the identification of some nematode species, also make this procedure the perfect candidate to become a standard method adopted in the context of a legal framework.

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CHAPTER 6

Inspection (I)

Case study: Microsporidians

This chapter is ready for submission as paper to the journal "Diseases of Aquatic Organisms" and we expect that the manuscript will have been submitted before the thesis defence.

Llarena-Reino, M., Abollo, E. and Pascual, S. Muscular microsporidians of anglerfish from NE Atlantic waters.

ABSTRACT

The presence of emergent visible parasites at commercial valuable fish species is increasingly causing problems at fisheries and seafood industries. Microsporidians have been previously reported to appear forming apparent xenomas in anglerfish species, but no effort has been done to simultaneously integrate epidemiological data, phenotypic, genotypic, and fine structural characterizations in the same parasite sample. In the present work, specimens of *Lophius budegassa* and *Lophius piscatorius* from NE Atlantic waters were sampled and examined to provide information about specific site of infection and demographic data of two groups of different sizes of xenomas present at both fish species. Histological descriptions and scanning and transmission electron microscopy were carried out on fresh spores of *Lophius budegassa* for ultra-structural studies. In both types of xenomas it was observed simultaneously the microsporidian genus *Spraguea* in the form of two different types of spores. Molecular analyses of both xenomas from the two fish species, based on the small subunit ribosomal DNA gene, were also performed to genetically support the morphological diagnostic provided.

KEYWORDS

Microsporidians; xenoma; *Lophius budegassa; Lophius piscatorius; Spraguea*

6.1. INTRODUCTION

Since the first description of microsporidian parasites in the soft nervous tissues of *Lophius* spp. (Thélonan 1895), several authors have described the appearance of diverse microsporidian genus in anglerfish species. Specifically, the presence of *Glugea, Nosema* and *Spraguea* genus have been described in both the white anglerfish *Lophius piscatorius* and the black anglerfish *Lophius budegassa*. Doflein (1898) firstly described *Glugea lophii* as a microsporidian infecting *L. piscatorius.* Some years later, Weissenberg (1911) established the name of this parasite as *Nosema lophii* on grounds, related to the development of the different phases of the spores. Sprague and Vávra (1976) firstly used the term *Spraguea lophii* to replace *Nosema lophii.* Since then, several authors have described the presence of the microsporidian parasites *Spraguea* spp. in different species of the genus *Lophius* (Table 6.1).

Table 6.1. Comparison among published articles and the present work about the microsporidian parasites *Spraguea* spp. present in *Lophius* spp. (anglerfish species), including information about parasite species, host species, geographical location and diagnostic methodologies.

Year of publication	Title	Author/s	Target species	Microsporidian parasite	Fishing area	DNA studies	TEM studies	SEM studies	Histological studies	Demography
1979	Étude ultrastructurale de *Spraguea lophii* (Doflein, 1898), microsporidie parasite de la baudroie: essai d'interprétation du dimorphisme sporal	C. Loubès et al.	*Lophius piscatorius* and *Lophius budegassa*	*Spraguea lophii*	Mediterranean Sea (*L.b.*) & Atlantic Ocean (*L.p.*)		X		X	
1986	The ultrastructure of spores (Protozoa: Microsporida) from *Lophius americanus*, the Angler Fish	P.M. Takvorian & A. Cali	*Lophius americanus*	*Glugea americana*	NE Atlantic coastal region		X		(Phase contrast microscope)	
1998	*Tetramicra brevifilum* (Microsporida: Tetramicriidae) in a new fish host, *Lophius budegassa* (Spinola, 1807) in Spain	P.A. Maíllo et al.	*L. budegassa*	*S. lophii* and *Tetramicra brevifilum*			X			
2000	Ribosomal DNA sequences of *Glugea anomala, G. stephani, G. americanus* and *Spraguea lophii* (Microsporidia): phylogenetic reconstruction	C. Pomport-Castillón et al.	*L. piscatorius* and *L. budegassa*	*S. lophii*		X				
2000	Small subunit ribosomal DNA phylogeny of Microsporidia with particular reference to genera that infect fish	F. Nilsen	*L. piscatorius*	*S. lophii*		X				
2003	Fish Microsporidia: fine structural diversity and phylogeny	J. Lom & F. Nilsen	*L. piscatorius* and *L. americanus*	*Spraguea* spp.		X	X		X	
2004	A microsporidian parasite of the genus *Spraguea* in the nervous tissues of the Japanese anglerfish *Lophius litulon*	M.A. Freeman et al.	*Lophius litulon*	*S. lophii* and *Spraguea americana*	Japan	99.5%	X		X	
2005	Microsporidian xenomas in fish seen in wider perspective	J. Lom & I. Dyková	*L. piscatorius*	*S. lophii*					X	

2010	Influence of host biological features on macroparasites of the two European anglerfish species, *lophius piscatorius* and *lophius budegassa*, off North and Northwest Spain	L. Cañás et al.	*L. piscatorius* and *L. budegassa*	*S. lophii*	NW Iberian Peninsula					(Prevalence only)
2011	*Spraguea* (Microsporida: Spraguidae) infections in the nervous system of the Japanese anglerfish, *Lophius litulon*, with comments on transmission routes and host pathology	M.A. Freeman et al.	*L. litulon*	*Spraguea* spp.	Japan	X			X	
2012	Redefining the genus *Spraguea* based on ultrastructural and phylogenetic data from *Spraguea gastrophysus* n. sp., a parasite found in *Lophius Gastrophysus* from Brazil	G. Casal et al.	*Lophius Gastrophysus*	*Spraguea gastrophysus*	Brazil	X	X			
(Present work)	Muscular microsporidians of anglerfish from NE Atlantic waters	M. Llarena-Reino et al.	*L. piscatorius* and *L. budegassa*	*S. lophii*	S Ireland & N Iberian Penins.	X	X	X	X	X

The first detailed description of *S. lophii* from *L. budegassa* and *L. piscatorius* (Loubès et al., 1979) was carried out by Takvorian and Cali (1986) who observed significant ultra-structural differences in the *Spraguea* spores. A few years later, some studies demonstrated divergences in *Spraguea* microsporidians between phylogenies, based on morphology and molecular data (Baker et al., 1995 and 1997). However, high similarities were observed in genetic sequences among parasites of the genus *Glugea* and between *Glugea* spp. and *S. lophii* (Pomport-Castillón et al., 1997 and 2000). Recently, some authors have suggested the assignation of *Spraguea* to *Glugea americanus* (Pomport-Castillón et al., 2000; Nilsen, 2000; Lom and Nilsen, 2003) and also to the microsporidians found in the Japanese anglerfish *Lophius litulon* (Freeman et al., 2004). The only species of microsporidians described until 1986 in black anglerfish had been *S. lophii* (Canning and Lom, 1986; Lom and Dyková, 1992; Sprague et al., 1992). However, Maíllo et al. (1998) observed different sizes of parasitic cysts from different anatomical locations of the Mediterranean anglerfish, reporting a simultaneous infection by *S. lophii* and *Tetramicra brevifilum.* This bibliographic review highlights that although the presence of *Spraguea* spp. in anglerfish is well-known, they have also been noticed many discrepancies about the phylogeny. Preceding studies have demonstrated a lack of concise information about the number of existing *Spraguea* species (Lom and Nilsen, 2003; Freeman et al., 2004; Lom and Dyková, 2005) and the need of more complete works on this genus including simoultaneous studies on morphology, genetics and epidemiology.

This work has the aim of providing a global study of this microsporidian parasite from the two Atlantic anglerfish stocks, *L. budegassa* and *L. piscatorius*, including site of infection and visual appearance of parasitic infestation, demography of infection, and finally, phenotypic and ultra-structural descriptions supported by genotypic data.

6.2. MATERIALS AND METHODS

6.2.1. Sampling and parasite isolation

Two lots (fifty individuals each one) of black and white anglerfish, *L. budegassa* and *L. Piscatorius*, were caught between August and September 2009 in NE Atlantic waters, between Ireland and Spain (VIIj and VIIh subareas of FAO 27 fishing area, respectively). Fishes were frozen within a maximum of 12 hours after capture. Every specimen was thawed, measured, weighed and visually examined with the aim of detecting possible external lesions or parasites (Table 6.2).

Table 6.2. Biological data and information relative to capture of the fishes examined.

Fish species	*Date of sampling*	*Sampling area*	*Sampling depth (m)*	*Individuals (N)*	*Total length range (cm)*	*Total weight range (g)*
Lophius budegassa	08/07/2009	FAO 27 (ICES) VIIj	134-167	50	35.5-52.5	571-1909
Lophius piscatorius	09/30/2009	FAO 27 (ICES) VIIh	134	50	26-38	269-826

Then, heads of each fish were removed to be individually observed looking for the presence of parasites. The musculature of every specimen was divided into muscular regions for being detailed inspected by candling. The detected xenomas bunches were isolated after reporting their specific position within the fish to be observed and photographed for further taxonomic assignment, and then recorded and preserved in 70% ethanol. Examined flesh of each individual was digested in artificial pepsin solution at constant pH and temperature, in an ACM-11806 Magnetic Stirrer with thermostatic heating Multiplate, following Llarena-Reino et al. (2013). Digested samples were sieved to retain hidden parasites, which were processed as previously described.

6.2.2. Histology and light microscopy

Xenomas selected for histological studies were fixed in 10% formaline for 24 hours before being dehydrated by gradient series of ethanol. Subsequently, they were immersed in liquid paraffin wax, sectioned into 6 µm thick slices and stained with haematoxylin and eosin. Finally, sections were observed under optical microscopy (Nikon Eclipse 80i).

6.2.3. Electron microscopy

A total of 10 fresh *L. budegassa* individuals from the same fishing area were necropsied without being frozen after capture. Thus, selected cysts in fresh condition were prepared for Scanning Electron Microscopy (SEM). Cysts were washed in 0.1M cacodylate buffer (pH 7.4), then sectioned on a wax plate with 2.5% glutaraldehyde in cacodylate buffer. They were immediately collected and preserved at 4°C for 2-4 hours. All spores in the contents were passed through a filter of 40 µm, sonicated during 5 minutes, and passed through another filter of 5 µm. Under a fume cupboard, samples were dehydrated in a gradient series of acetone and drained using the critical point-dried desiccators. Finally, the spores were mounted on stubs, gold-coated and observed in a Philips XL30 scanning electron microscope for a detailed external description.

For Transmission Electron Microscopy (TEM), selected cysts were fixed in 5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) during 10 hours. Then, they were immersed in the same cacodylate buffer for 2-12 hours and post-fixed with 2% OsO_4 in the same buffer during 5 hours. Subsequently, cysts were dehydrated by 2 gradient series of ethanol finishing with 2 series of 15 minutes of propylene oxide. They were immersed in Epon resin (Luft 1961) with propylene oxide for 36 hours, and polymerized at 60°C during 48 hours. Ultrathin sections of 70-90 nm were obtained with a Leica Reichert Ultracuts ultramicrotome, and stained with uranyl acetate and lead citrate (Reynolds, 1963). After selecting the best sections, observations with a JEOL JEM 1010 transmission electron microscope at 80 kV were carried out for a full description of the internal structure of spores.

6.2.4. Demography of infection

After histological and ultra-structural studies of both types of xenomas, and once collected all data about parasites and sites of infection, demographic values as prevalence (P), mean intensity (I) and mean abundance (A) of infection were determined for parasites at both fish species following Bush et al. (1997) and Rózsa et al. (2000). Non-parametric tests were used to determine the statistical significance of the relationships between number of xenomas and size-weight of the fish.

6.2.5. Molecular analysis

6.2.5.1. DNA extraction and PCR amplification

With the purpose of genetically identify microsporidians from the two *Lophius* species, both types of xenomas found in the frozen lots were selected and prepared for molecular analyses based on the small subunit ribosomal DNA gene.

DNA extraction process was carried out with the commercial kit NucleoSpin® Tissue Kit (Macherey–Nagel GmbH, Düren, Germany) according to the manufacturer's recommendations. A partial region of the 18S rRNA gene was amplified using the primers V1f (5'-CACCAGGTTGATTCTGCC-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Nilsen, 2000; Vossbrinck et al., 1993). All PCR mixtures were performed in a total volume of 25 µl containing 1 µl of genomic DNA (150-200 ng), PCR buffer at 1x concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM each primer and 0.025 $U.\mu l^{-1}$ Taq DNA polymerase (Roche Applied Science, Germany). The cycling protocol was 2 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, followed by 7 min at 72°C. All PCRs were carried out in a TGradient thermocycler (Biometra) and a negative control (without DNA) was included for each set of PCRs. PCR products were separated on a 1.5% agarose gel in 1x TAE EDTA buffer, stained with 5 µL/100 mL RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology), and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories).

6.2.5.2. DNA cloning and sequencing

PCR products were ligated into cloning vector pCR™4-TOPO® TA for 15 min at room temperature and transformed into *E. coli* One Shot Top 10F' Chemically Competent cells (Invitrogen Life Technologies) following the manufacturer's instructions. Transformed cells were screened by PCR using the vector's primers M13 forward (5' GTA AAA CGA CGG CCA G3') and reverse (5' CAG GAA ACA GCT ATG AC 3'). PCR profile consisted of an initial denaturation at 94**°**C for 5 min, 35 cycles with initial denaturation at 94**°**C for 30 sec, annealing at 55**°**C for 45 sec, and elongation at 72**°**C for 90 sec, final elongation at 72**°**C for 7 min. The positive clones were cleaned for sequencing using ExoSap-IT (USB Corporation) enzyme, as supplied by the manufacturer. PCR products were sent to Secugen S.L. Company (Madrid) for sequencing.

6.2.5.3. Phylogenetic inference.

Sequence chromatograms were analysed using CromasPro version 1.41 Technelysium Pty LtdA. All generated sequences were searched for similarity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Center for Biotechnology Information (USA). Sequence sets for 18S rRNA gene were aligned in ClustalW multiple alignment of MEGA6 programme (Tamura et al., 2013) under default parameters. Alignments were used to construct phylogenetic tree using maximum likelihood (ML) and the best nucleotide substitution patterns for ML trees were selected based on the analyses of best-fit models in MEGA6. The ML trees were computed using the Jukes Cantor model of evolution with a bootstrap test (1000 replicates).

6.3. RESULTS

6.3.1. Macroscopic examination

Microsporidians were found in both anglerfish species, forming bunches of xenomas on the medulla oblongata of the hindbrain (hereafter Zone A), and smaller formations in the nervous tissues along the length of the vertebral column (hereafter Zone B) (Fig. 6.1). Xenomas sizes were variable (from 1.6 to 5.2 mm diameter), the bigger ones being located at Zone A, and the smaller ones at Zone B. Xenomas located in caudal areas of Zone B, frequently showed a harder and calcified consistency.

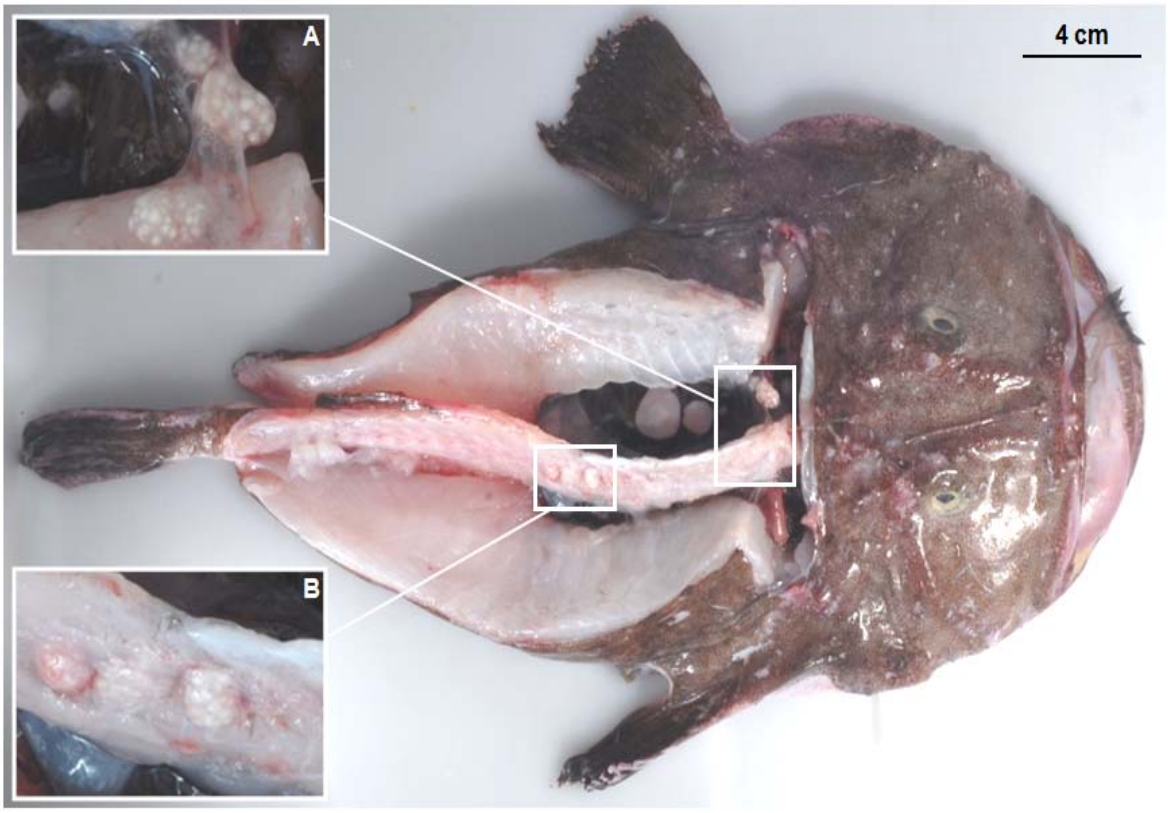

Figure 6.1. Location of microsporidian xenomas infecting nervous tissues of *Lophius budegassa*. A dorsal dissection of the fish shows some parasitic cysts situated in Zones A and B.

6.3.2. Histology and light microscopy

Stained histological sections of rounded clusters of xenomas revealed a different intensity staining of the parasite mass at the margin of the host cell (*Nosemoides*-type spores), from that in the centre (*Nosema*-type spores), as described by Loubès et al. (1979) and Lom and Dyková (2005) (Fig. 6.2). During the reaction that host's immune system sets against parasites, several stages of xenomas were observed coexisting inside the host cells; from earliest cyst-like forms to the most advanced granulomatous parasitic lesions. In our histological studies, encysted and highly developed phases of xenomas have been the most observed stages. Semi-thin slides evidenced granulomas with a layer composed of collagen covering them and an external wall formed by the typical eosinophilic cells that contribute to the reconstruction process of the affected host cells (Fig. 6.3).

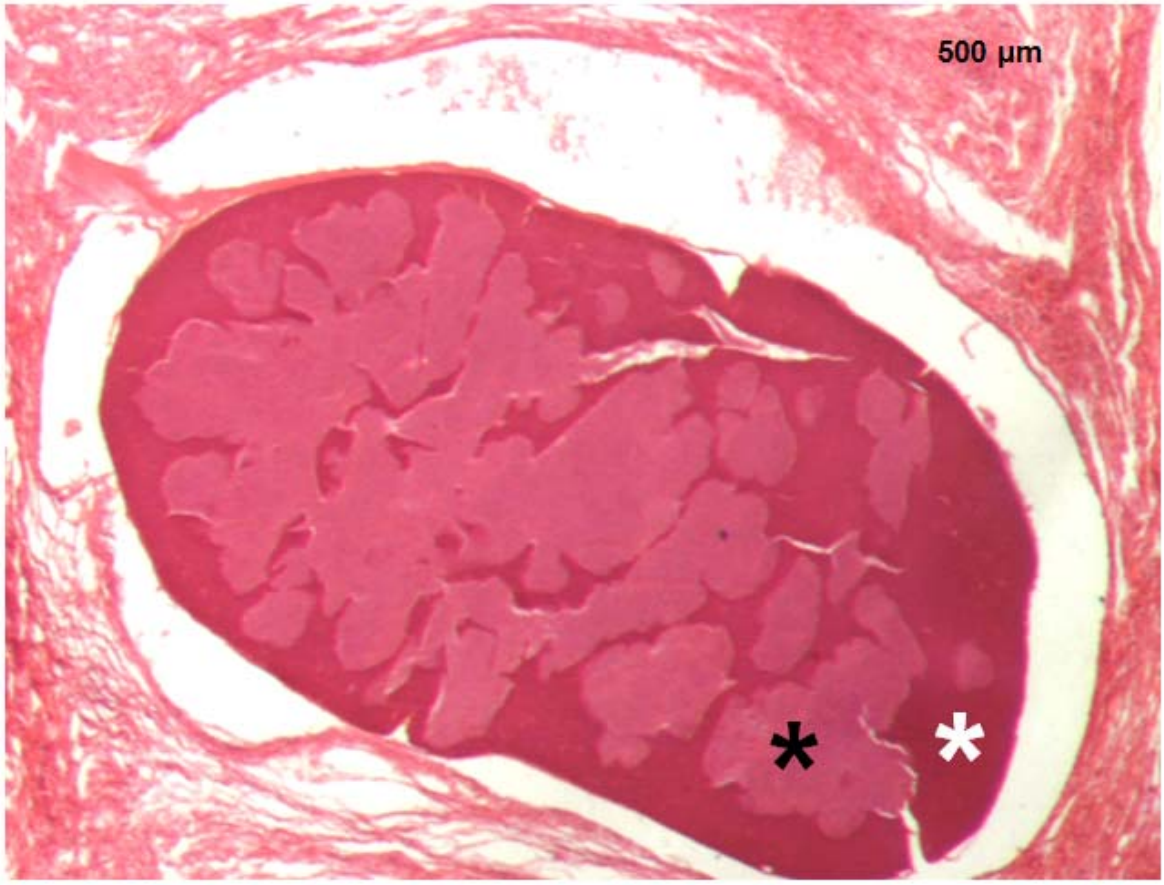

Figure 6.2. Light micrograph of a mature xenoma of *Spraguea* sp. (from Zone A) partially transformed into granuloma, infecting nervous cells of *Lophius budegassa. Nosema* and *Nosemoides*-types of spores (black and white asterisks respectively) are evidenced by the two different staining intensities within the xenoma. H&E staining.

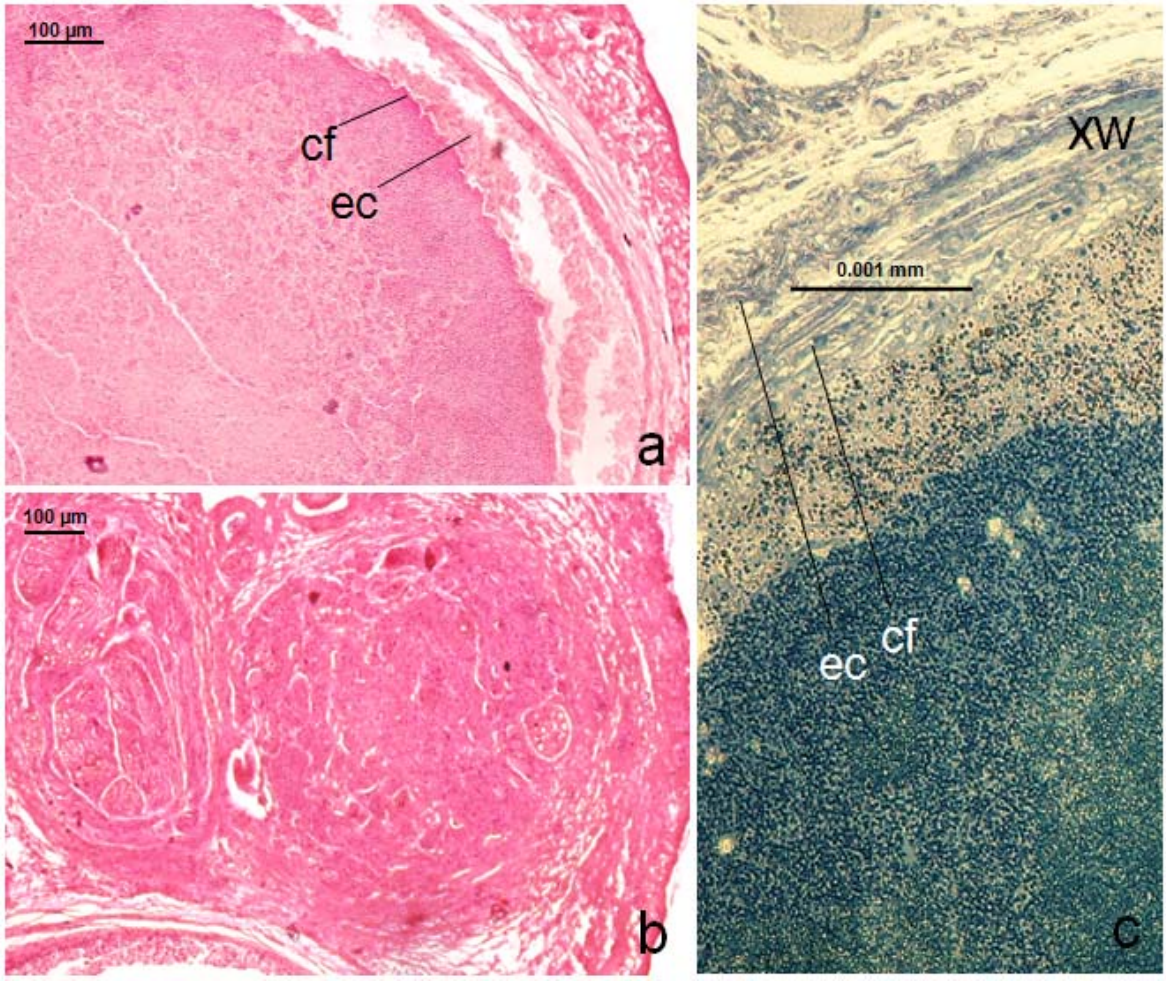

Figure 6.3. (a-c). Light micrographs of fish microsporidian (*Spraguea* sp.) xenomas from Zone A, infecting nervous cells of *Lophius budegassa.* **a.** Xenoma of *Spraguea* sp. partially transformed into granuloma. A layer composed of collagenous fibers (cf) and an eosinophilic cells wall (ec) situated externally are covering it. H&E staining. **b.** Granulomatous lesion from a *Spraguea* xenoma. H&E staining. **c.** Toluidine blue-stained semi-thin section showing the xenoma wall (xw) of a granulomatous lesion from a *Spraguea* xenoma.

6.3.3. Electron microscopy

SEM allowed the visualization of spores with two different shapes and size ranges inside both types of xenomas; more widespread spores with a notable curvature (1.6 x 3.8 µm of average length), and rounded, shorter and filled type spores (2.1 x 2.9 µm of average length) (Fig. 6.4).

TEM studies firstly demonstrated predominantly mature spores in the different selected and sectioned xenomas. However, the evolution process of the spores evidenced the presence of other stages; some dividing and developing sporoblasts and early spores were also found among mature spores. During TEM observations, division of sporoblasts in two early sporoblasts and a progressive formation of developing sporoblasts were observed. At those stages the nucleus was formed, and typical electron-lucent vesicles

that would later become internal structures (organs) of the spores began to be noticed. During that period it was also observed that the outer wall gets thicker, and the rounded ridges that later are going to cover the external surface of the spores acquires a more noticeable appearance. Finally, before the mature spore stage go to term, early spores appeared with a developing sporoplast including the posterior vacuole, as well as a polar filament formed by turns whose interiors show electron-dense cores (Fig. 6.5). Otherwise, it was observed that the wall of mature spores is being formed by an electron-lucent endospore and an electron-dense-exospore which contained the rounded ridges covering the surface of monokaryotic spores of *S. lophii* (Fig. 6.6).

Two differentiating features observed among spores were the numbers of nuclei present in the polaroplasm and the quantity of turns that the polar filament shows. As Fig. 6.7 demonstrates, in this study many mature spores included two nuclei seeming to have diplokaryotic morphology (*Nosema*-type spores) and up to 11 turns of the polar filament. On the other hand, spores with one central nucleus (monokaryotic typical structure or *Nosemoides*-type spores) were seen in smaller proportion, showing no more than 5 turns of the polar filament. Concerning other internal structures that could be described in TEM images (Fig. 6.8), anterior ends of the spores could be perceived with an anchoring disc that evidenced a significant reduction of the endospore at that level. From there, the manubroid part of the polar tube and the laminar polaroplast around it, were located. To the rear, this laminar polaroplast became a tubular structure (tubular polaroplast) more closely associated with the polar tube, which does not concern the width of the spore (Freeman et al., 2004). As the polar tube goes deep into the spore it becomes polar filament, presenting 3 to 5 coils in uninucleated spores and 3 to 11 turns in binucleated spores, all positioned in the same plane (Fig. 6.7). Moreover, the posterior vacuole, located in the rear end of spores, usually enclosed the electron-dense inclusion bodies that are formed during the sporoblast stage, which can range from small to large accumulations.

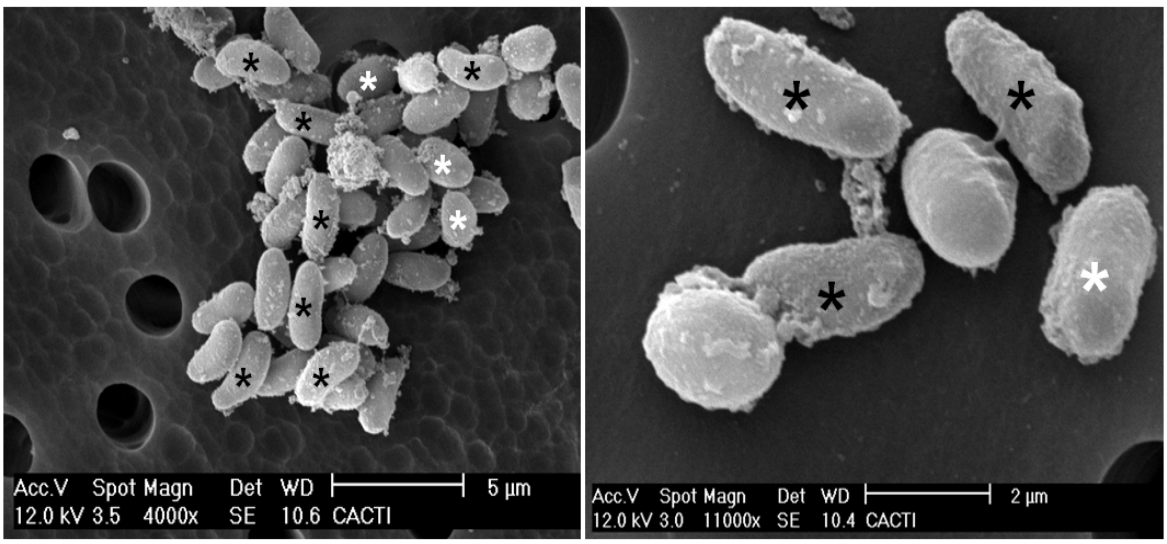

Figure 6.4. Mature spores of *Spraguea* sp. from Zone B in *Lophius budegassa*, observed under scanning electron microscope. Two different spores; a widespread and curved type (black asterisks) and a rounded, shorter and filled variety (white asterisks).

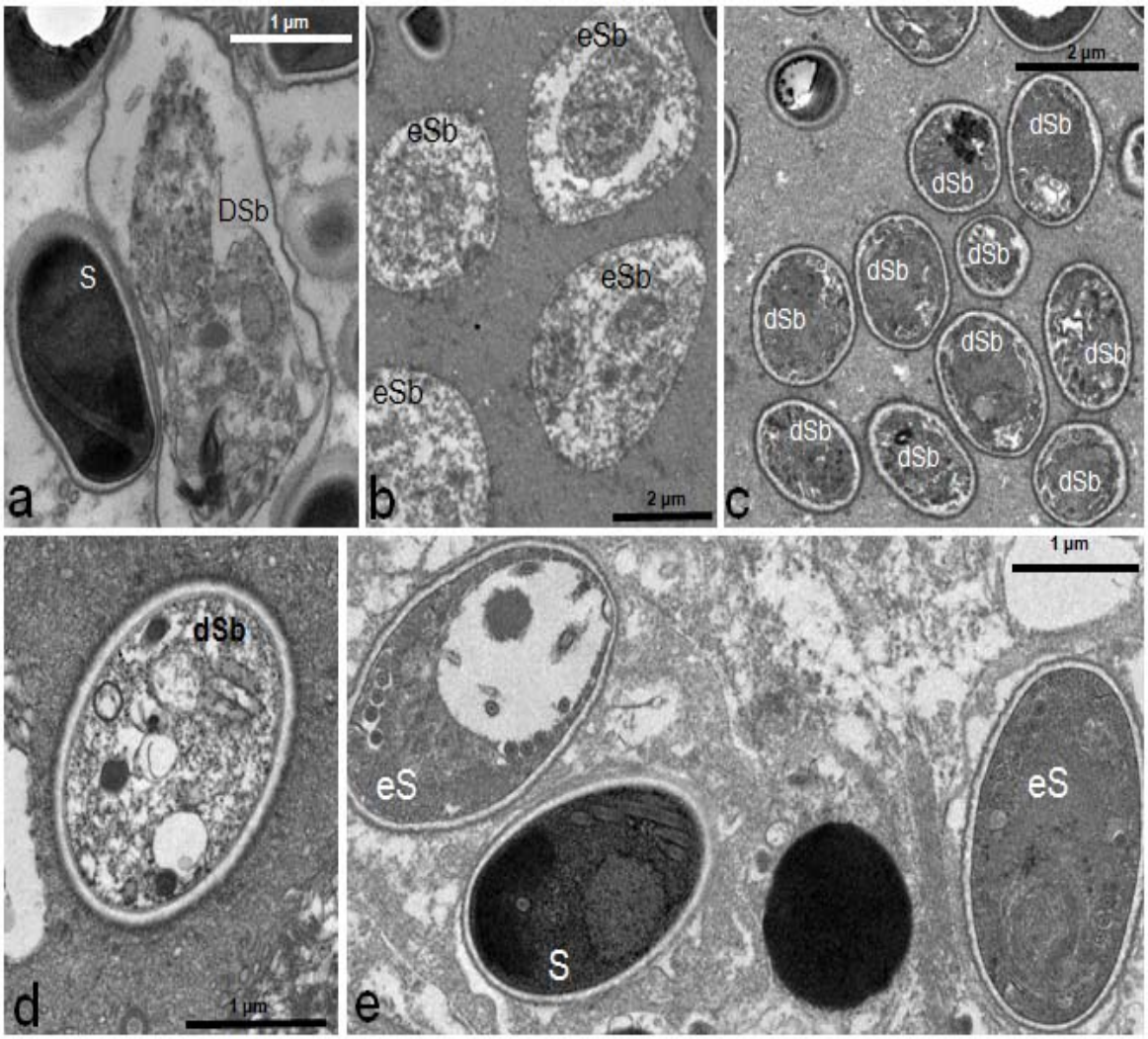

Figure 6.5. (a-e). Sectioned *Spraguea* xenomas from Zones A and B of *Lophius budegassa*, observed under transmission electron microscope. **a-c.** After the dividing sporoblasts (DSb) get separated in two, the resulting early sporoblasts (eSb) change to become developing sporoblasts (dSb). Spore (S). **d-e.** Developing sporoblasts continue their evolution until their structures resemble an early spore (eS), and finally a mature spore.

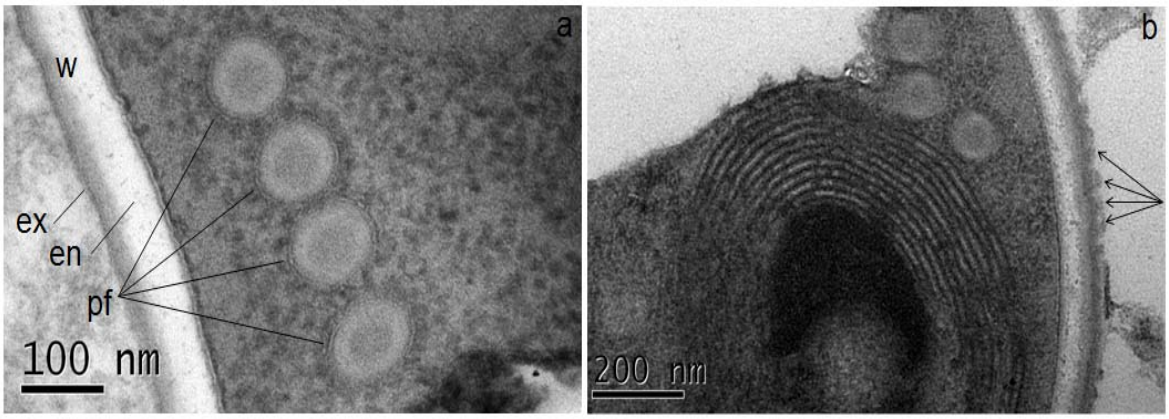

Figure 6.6. (a-b). Detail of the wall of mature spores of *Spraguea* sp. from Zones B and A respectively of *Lophius budegassa,* observed under transmission electron microscope. **a.** Wall formed by two layers; exospore (ex) and endospore (en). Four turns of the polar filament (pf) composed by an external double-layer and showing electron-dense cores inside each section. **b.** Transverse section of a spore which shows the typical rounded ridges that cover the exospore surface of monokaryotic spores of *Spraguea* sp. (arrows).

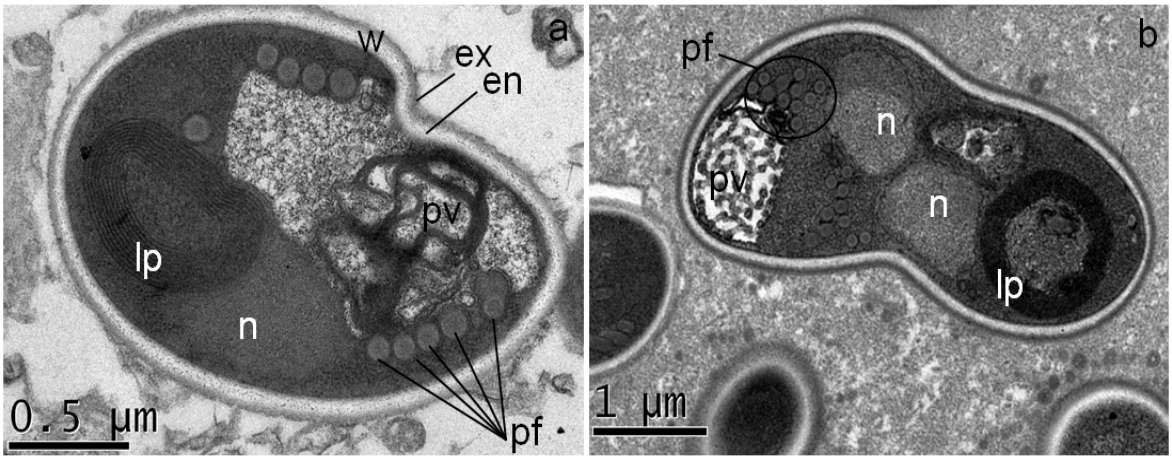

Figure 6.7. (a-b). Mature spores of *Spraguea* sp. from Zones A and B (figures a and b, respectively) of *Lophius budegassa,* observed under transmission electron microscope. Monokaryotic (one nucleus) and diplokaryotic (two nuclei) type spores showing five and eleven turns of the polar filament (pf), respectively. Posterior vacuole (pv), laminar polaroplast (lp), nucleus (n), exospore (ex), endospore (en), wall (w).

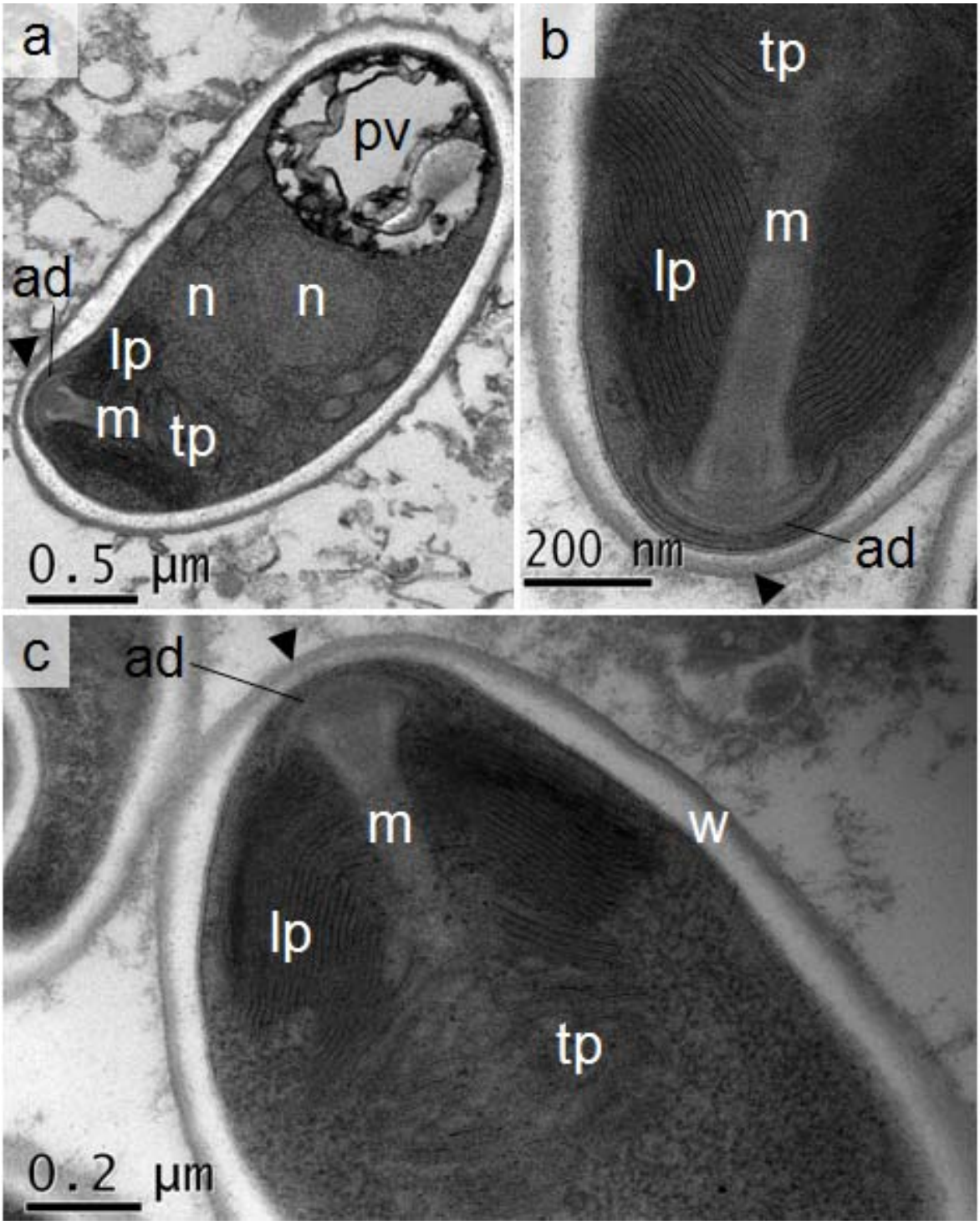

Figure 6.8. (a-c). Transmission electron micrographs of microsporidian mature spores of *Spraguea* sp. from Zones A (figure a) and B (figures b and c) of *Lophius budegassa.* Anterior ends of the spores (black arrowheads) show the anchoring disc (ad), the manubroid part of the polar tube (m), tubular and laminar parts of the polaroplast (tp, lp). The posterior vacuole (pv) is located in the posterior zone of the spores.

6.3.4. Demography of infection

Demographic values of microsporidians were determined for both fish species (Table 6.3). The infection has been observed to be more relevant in *L. budegassa*, affecting equally both Zone A and B, and revealing higher prevalence and abundance than in *L. piscatorius*. White anglerfish showed higher values of infection when parasites were affecting both Zone A and B, than only Zone B. Additionally, black anglerfish showed most of the xenomas bunches in Zone B (82.6%), and only few ones in Zone A (17.4%). By contrast, white anglerfish had most of the microsporidian formations in Zone A (75%). There were not statistical relationships (Pearman correlation and Mann-Whitney tests showed $p>0.05$) between the number of xenomas and the size or weight of fishes.

Table 6.3. Demographic values of microsporidia infection determined by anatomical region for *Lophius budegassa* and *Lophius piscatorius*.

Fish species	*Individuals (N)*	*Anatomic region*	*Prevalence (% ± CI)*	*Mean Intensity (± SD)*	*Mean Abundance (± SD)*
Lophius budegassa	50	Body	24 ± 5.9	1.58 ± 0.96	0.38 ± 0.78
		Body and head	24 ± 5.9	1.91 ± 1.32	0.46 ± 1
Lophius piscatorius	50	Body	2 ± 1.9	2 ± 0	0.04 ± 0.28
		Body and head	14 ± 4.8	1.14 ± 0.35	0.16 ± 0.44

6.3.5. Molecular identification

The 18S sequence of *Spraguea* parasite infecting black and white anglerfish obtained in this study showed 100% identity among them. BLAST searches showed high identities values (99%) to *Spraguea lophii* (AF033197; AF104086), *Spraguea* sp. from *Lophius litulon* (AY465878) and *Spraguea gastrophysus* from *Lophius gastrophysus* (GQ868443). The phylogenetic tree inferred using ML method showed that the sequences obtained in this study were placed in a clade with high bootstrap value (99%) with other members of the genus *Spraguea* infecting *Lophius* species (*S. lophii, S. americanus, S. gastrophysus* and *Spraguea* sp.) (Fig. 6.9). It is noteworthy that species of the genus *Spraguea* infecting *Seriola* species were placed in other clade forming a paraphyletic group. *Spraguea* sp. from *Seriola quinqueradiata* and *S. dumerili* clustered with species of the gena *Microgemma* and *Tetramicra* with bootstrap values of 86%.

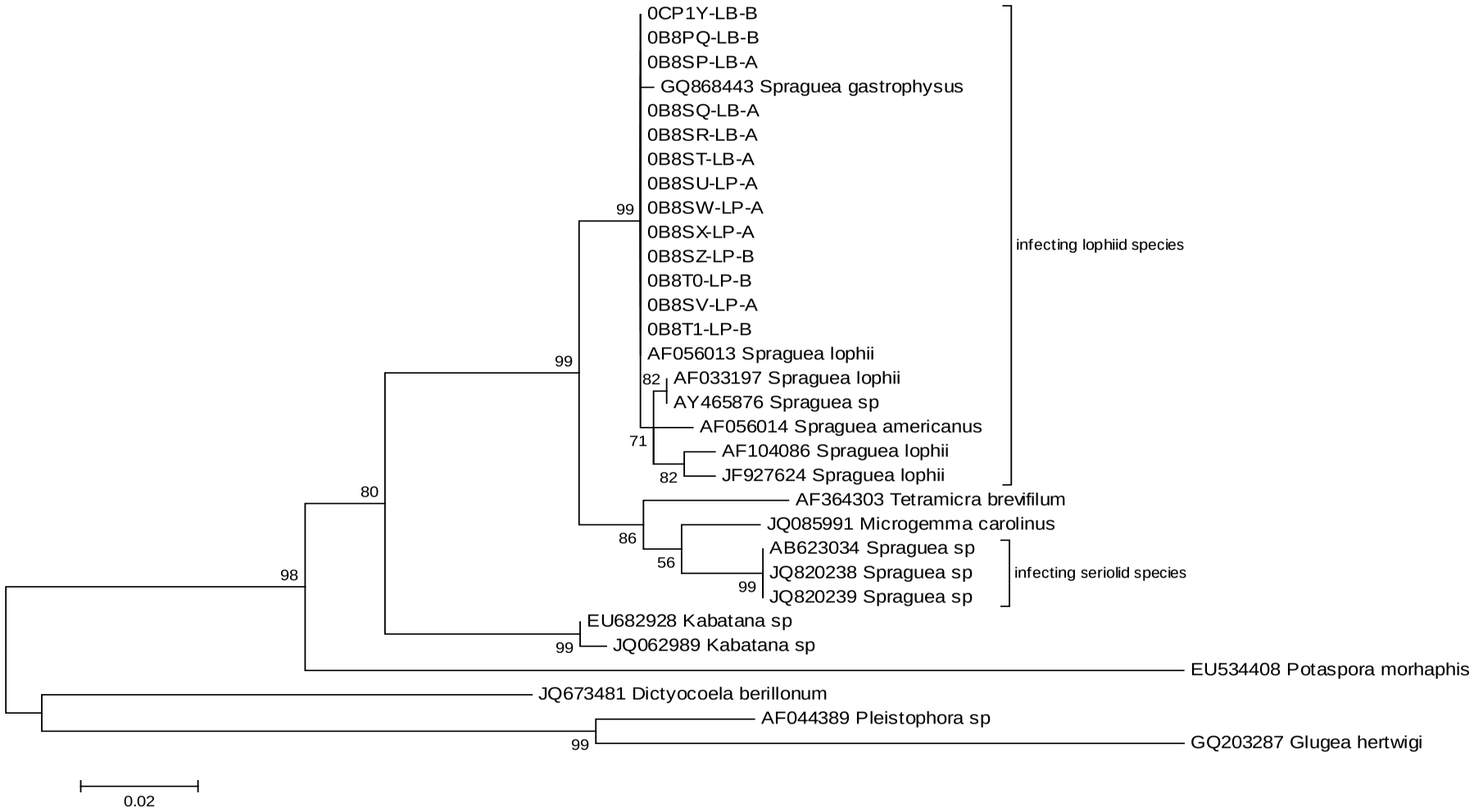

Figure 6.9. Phylogenetic position of *Spraguea* sp. infecting *Lophius piscatorius and L. budegassa* inferred from the maximum-likelihood (ML) analysis of partial 18S rDNA sequences. Numbers at branch nodes indicate bootstrap confidence values in percent. *Spraguea* samples analysed are coded as "a five-digit alphanumeric code - *Lophius* spp. (LB or LP) - cyst location in fish (Zone A or B).

6.4. DISCUSSION

6.4.1. Parasite characterization

In 2011, Freeman et al. discussed about a clearly pattern of microsporidian infection which revealed that medulla oblongata (Zone A) strongly seemed to be the primary site of infection. As they also exposed, some nerves as the spinal, trigeminal and vagus (throughout its length) appear as the second region of infection when the contamination on the medulla becomes more serious. As the present study reveals, white anglerfish presented almost the total number of bunches in Zone A, and it was clearly shown to be the primary site of infection, with higher values of prevalence and abundance than mixed-infected fishes (Zones A+B). This pattern of infection was not found in black anglerfish, in which almost the total number of bunches was found in Zone B of parasitized fishes. However, for this species the number of Zone B-infected fishes was the same than the quantity of mixed-infected hosts. Therefore, accordingly with Freeman et al. (2011) our results also suggest a tendency to this pattern of infection, at least observed in *Lophius piscatorius.* Historically, the only published morphological comparative study on *Spraguea,* specifically on *S. lophii,* affecting both *L. piscatorius* and *L. budegassa,* was that from Loubès et al. (1979). In that work, which included the first detailed ultrastructure report of this microsporidian genus, authors analyzed a total of 24 individuals (11 *L. piscatorius* and 13 *L. budegassa,* from Atlantic and Mediterranean waters, respectively), and provided the spore dimensions: 1.5x3.5 µm for monokaryotic, uninucleated or *Nosemoides* spores and 1.25x4 µm for diplokaryotic, binucleated or *Nosema* ones. The present work shows clear differences from those preceding dimensions. Some authors (Lom and Dyková, 1992; Maíllo et al., 1998) attributed the existence of differences between spore sizes in microsporidians, to the observation of the parasite in atypical hosts. However, heterogeneity in spore dimensions reported by Loubès et al. (1979) and by the present work reflects that this is not likely to be the case, since previously described anglerfish species parasitized by microsporidians have demonstrated not to be atypical hosts. In addition, molecular characterization of isolated parasites from both NE Atlantic anglerfish species here inspected, have revealed a high sequence homology between samples of the four different types of xenomas detected under the visual scheme. This result contrasts with that of Maillo et al. (1998) who reported the simultaneous infection by two microsporidians in Mediterranean anglerfish corresponding with two different sizes of cyst types observed in *L. budegassa*. Furthermore, Pomport-Castillon et al. (2000) summarized the presence of *S. lophii* as single infection during comparative phylogenetic studies of microsporidians affecting anglerfish.

Ultra-structural studies of xenomas extracted from NE Atlantic black anglerfish, *L. budegassa*, revealed a similar cytoplasmatic configuration in spores among both types of xenomas (from Zones A and B), corresponding to previous descriptions of *Spraguea* genus. It can be concluded that this morphological variability or phenotypic plasticity of spores may be due to evolutionary changes or different forms of appearance of this parasite, or to different sexual phases of reproduction responding to external environmental conditions (Loubès et al., 1979). Similarly, in monokaryotic spores of *S. lophii* observed in *L.*

piscatorius and *L. budegassa*, Loubès et al. (1979) described 5-6 turns of polar filament while in diplokaryotic spores the quantity of defined turns was 3-4. Regarding this morphological characteristic, results from the present work reveal a minimum and maximum numbers of turns in the polar filaments which contrast with those previously published. Consequently, these results also differ to those that affirm that all uninuclear forms have a comparable number of turns in the polar tube (Freeman et al., 2004). In any case, the differences in some morphological characteristics of diagnostic characters of the spores, as the number of observed nuclei, quantity of turns of the polar tube, and spores average length, should be taken into account during diagnoses when using TEM, and also with the purpose of carrying out a more detailed genetic analysis. In addition, it should be necessary to take these findings into consideration to determine the reason why morphological results reported here differ from those previously published, and why dimorphism seems to be an exclusive characteristic of the *Spraguea* infections affecting only *L. piscatorius* and *L. budegassa* (European lophiid species) and not American or Japanese anglerfishes (Casal et al., 2012).

6.4.2. Impact on fish

Based on severe lesions observed, *Spraguea* spp. has been referred to induce hypothetical pathogenic effects in lophiids. Freeman et al. (2004) concluded that despite the poor condition and disorders found in the inspected infected fishes there is not convincing scientific evidence to affirm that *Spraguea* spp. produce disease. Freeman et al. (2011) highlighted that *Spraguea* infections in the hindbrain region of *Lophius* spp. are generally not pathogenic, even in heavily infected hosts. However, they sustained the possibility that serious pathogenicity could be caused by the infection of other nerves, due to the absence of such sites of infection identified during their study. In the present work, the high number of xenomas observed along the spinal, trigeminal and vagus nerves, and specially the presence of harder and calcified consistency of xenomas located in the caudal areas of Zone B, could impair the swimming movements of the tail fin. Furthermore, the higher size of xenomas situated in Zone A also could hamper the central nervous system and vital organs of fishes by pressing the medulla oblongata.

The commercial impact of *Spraguea* spp. xenomas in fish quality is also an industrial concern, mostly due to the applicability of Regulation EC 178/2002 which establishes that for reasons of contamination anglerfish infected with visible parasites is unfit for human consumption. Furthermore, on the basis of a possible zoonotic potential mentioned by Canning and Lom (1986), it would be desirable to enlarge studies that provide information about their potential effects on the consumers' health and the consequences of severe parasitizations in the short term. Monitoring actions and proactive self-inspections, besides preventive and corrective measures, should be implemented to guarantee safer and high quality standard products to final consumers.

6.5. ACKNOWLEDGEMENTS

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CHAPTER 7

Inspection (II)

Case study: Anisakids

Epidemiology of *Anisakis* spp. larvae in fresh fish flesh from Vigo markets

ABSTRACT

A total of 1910 individuals grouped into 25 fresh fish lots belonging to 13 species from fish establishments placed in market squares of Vigo (Galicia, Spain), were seasonally processed by the press method and examined for the presence of anisakid larvae in muscle. An UV-Cabinet was used as a high-throughput screening tool for fresh fish flesh inspection. Flatfish and coastal species were free of anisakids or had no significant infection values. However, fish species which are known to be keystones in trophic webs of fishing grounds (i.e., the blue whiting, *Micromesistius poutassou* and the European hake, *Merluccius merluccius*) showed by far the highest demographic infection values. For both fish species, densities of anisakid infection exceed the international standards for accepting fish products, which means that those fish lots have demonstrated substantive weaknesses in health prognostic factors. Compared results with previous seroprevalence data outline the great importance of prophylactic measures at the consumer level, and the need for a monitoring programme for unhealthy and anaesthetic anisakids in the flesh of *M. merluccius* and *M. poutassou* which guarantee consumer acceptance of both seafood products.

KEYWORDS

Anisakids; fish; *Merluccius merluccius; Micromesistius poutassou*; press method; UV

7.1. INTRODUCTION

As a consequence of recent medical recording of gastrointestinal disorders and allergic reactions, both dead and alive nematode parasites of Anisakidae are considered an emergent health hazard in seafood products (Baeza et al., 2001; Audicana et al., 2002; EFSA, 2010), especially in some Asian and southern European countries where raw, lightly salted or marinated fishes are component of the daily diet (Maggi et al., 2000; Adams et al., 1997). Anisakids are also well-known to impair the commercial quality of fish infected tissues, due to pathological changes that may range from mild to severe, as nodules in belly flaps, melanized capsules in fillets, milky flesh or muscle fibber destruction (Vidacek et al., 2009), hemorrhages in the vent areas (Beck et al., 2008), or gross inflammation in fishes from aquaculture industry (Hauck and May, 1977; Marty, 2008). The anaesthetic appearance of heavily contaminated fishes also contributes to consumer rejection, mostly associated with parasite motility in fresh fish (Pascual et al., 2010), and to decrease commercial value of seafood products (Fischler, 2002).

Despite the refused or downgraded fish flesh caused by the presence of anisakids, which in fact are tending to spoil consumers' appetites, most of the surveillance programs implemented at seafood industries have been focused on inspections by making indirect observations at anisakids in the fish viscera and gut cavity.

These “non-destructive” methods of parasite inspection, as gross visual observation and candling, the commonly recommended detection procedures to be carried out by the fish companies, are most likely due to the legal mandatory of European Union regulation 91/493/EEC and Commission Regulation (EC) 2074/2005. According to these and other fish quality regulations, marine specimens intended for human consumption containing visible parasites found during industrial self-controls, cannot be marketed or sold. In the case of whole fish, these inspection practices have been considered inadequate because in the best case parasite burdens are being under detected in industrial conditions (Levsen et al., 2005). These self-control methods are limited by the fact that the resulting information is based on estimations with no statistical confidence (Llarena-Reino et al., 2012). Although most anisakid larvae are found in the viscera, mesentery and gonads of the fish (Davey, 1972; Vidacek et al., 2009), they are also usually present in a lower amount in the flesh (Wharton et al., 1999; Llarena-Reino et al., 2012), especially in the belly flaps (Wooten and Smith, 1976; Berland, 2003) and at times deeply in the epaxial musculature (Smith, 1984), which in most cases is sufficient to affect food quality and safety. Even though liver and gonads can be found in local markets as well, their consumption is relatively low compared with the flesh of commercial fresh fishes. Moreover, Levsen et al., revealed in 2005 that only 7 to 10% of nematode larvae present in fillets was detected by candling when comparing with “destructive” detection methods as pepsin-HCl digestion (Llarena-Reino et al., 2013a) or UV examination of deep-frozen fillets (Karl and Leinemann 1993; Lunestad, 2003). Therefore, in contrast to non-destructive procedures, invasive fish inspection methods are considered “better” or “truer” because they allow direct examination of flesh parasites and their spread in the edible part of fish. Identification of *Anisakis* larvae in fish products utilizing their auto-fluorescence after the excitation of the sample with a 365 nm wavelength of UV-light source in dark conditions, is a method based on the visual inspection of flattened/pressed and deep-frozen fish fillets or viscera under UV-light. As previously described by some authors (Pippy, 1970; Karl and Leinemann, 1993; Lunestad, 2003), their benefits are so great that many approaches to implement its application at industry have been carried out in the recent years (Levsen and Lunestad, 2010; Sivertsen et al., 2011 and 2012).

The purpose of this research has been to accomplish the plea from the European Food Safety Authority (EFSA, 2010) regarding to provide more epidemiological available information for potentially consumer hazardous parasites, by studying the efficiency and reliability of the press technique and visual inspection under an UV-light source for the detection of nematode larvae in the flesh of commercially important pelagic fishes. Herein we report the epidemiological results on anisakids, after inspection of fresh fish marketed in two different sampling seasons in Vigo (Galicia, Spain).

7.2. MATERIALS AND METHODS

A total of 1910 fresh individuals distributed in 25 lots corresponding to 13 fish species (*Pegusa lascaris, Dicologlossa cuneata, Brama brama, Trisopterus luscus, Lepidorhombus* spp., *Trachurus trachurus, Solea* spp., *Micromesistius poutassou, Merluccius merluccius, Zeus faber, Sardina pilchardus, Diplodus sargus* and *Engraulis encrasicholus)* were seasonally, in autumn and spring, bought in fish establishments placed in market squares of Vigo (Galicia, Spain), and examined for the presence of anisakid larvae in muscle. Only one of the species present in autumn, *Engraulis encrasicholus,* was absent in the spring sampling. Immediately after procurement, whole fish were measured (fork length ±5 mm) and weighed (±1 g), and subsequently attributed to the following freshness classification groups: extra, A and B, according to international standard Council Regulation (EC) 2406/96 and Spanish Royal Decree 331/1999. All this information was recorded and is summarized in Table 7.1.

Table 7.1. Data from the fish lots studied including fork length and weight ranges, the number of specimens belonging to three freshness classification groups (Extra, A and B) and demographic values of anisakid infection (P, A, I and D).

Season	Fish species	N	Prevalence (% ± CI)	Mean Abundance (± SD)	Mean Intensity (± SD)	Density	Mean fork length [range] (cm)	Mean weight [range] (g)	EXTRA (N)	A (N)	B (N)
Spring	*Pegusa lascaris*	62	0	0	0	0	24.6 [20-36.5]	177 [108-304]	24	25	13
	Dicologlossa cuneata	23	0	0	0	0	15.1 [12.5-17.5]	39 [23-62]	7	14	2
	Brama brama	84	2.38±1.63	0.02±0.11	1±0	0.03	43.3 [38-50]	789 [534-1287]	0	43	41
	Trisopterus luscus	84	0	0	0	0	21.85 [15.5-29.5]	116 [39-262]	12	32	40
	Lepidorhombus spp.	84	11.9±3.46	0.17±0.51	1.4±0.7	1.08	27.8 [19-33]	153 [48-245]	0	22	62
	Trachurus trachurus	84	26.19±4.7	0.55±1.62	2.09±2.64	2.09	30.2 [19-40]	264 [55-573]	10	73	1
	Solea spp.	86	0	0	0	0	31.56 [24-46]	308 [126-1095]	7	23	56
	Micromesistius poutassou	84	25±4.63	0.75±2.53	3±4.42	13.34	21.7 [16-28.5]	57 [18-113]	0	71	13
	Merluccius merluccius	83	65.06±5.13	4.35±6.6	6.69±7.21	5.42	46 [24-76]	802 [77-2243]	6	47	30
	Zeus faber	84	30.95±4.94	0.71±1.35	2.31±1.57	1.18	33 [20.5-49.5]	604 [128-2125]	0	16	68
	Sardina pilchardus	84	2.38±1.63	0.02±0.15	1±0	0.49	17.9 [15.5-24.5]	49 [28-141]	47	23	14
	Diplodus sargus	84	0	0	0	0	31.3 [22-38.5]	579 [177-1090]	29	28	27
Autumn	*Pegusa lascaris*	25	0	0	0	0	23.9 [16.5-28.5]	146 [52-236]	0	12	15
	Dicologlossa cuneata	59	0	0	0	0	15.1 [13-17.5]	37 [20-68]	0	30	29
	Brama brama	84	2.38±1.63	0.05±0.34	2±1.41	0.05	44.4 [35-51]	949 [340-1500]	6	27	51
	Trisopterus luscus	84	1.19±1.16	0.05±0.44	4±0	0.45	20.8 [14-27.5]	105 [26-252]	3	12	69
	Lepidorhombus spp.	84	5.95±2.53	0.11±0.58	1.8±1.79	0.65	27.5 [19.5-38.5]	166 [62-464]	0	2	82

Trachurus trachurus	84	10.71±3.31	0.17±0.58	1.56±1.01	0.55	29.8 [13.5-42]	299 [22-672]	46	33	5
Solea spp.	84	0	0	0	0	29.5 [24-39]	260 [118-632]	0	15	69
Micromesistius poutassou	84	10.71±3.31	0.13±0.43	1.22±0.67	4.54	16.6 [13.5-20.5]	29 [17-51]	0	56	28
Merluccius merluccius	84	45.24±5.32	16.85±36.02	37.24±46.16	18.22	47.6 [25-77]	912 [110-2722]	6	28	50
Zeus faber	84	30.95±4.94	1.44±3.73	4.65±5.54	2.89	30.5 [19-47]	502 [97-1678]	0	14	70
Sardina pilchardus	84	0	0	0	0	19.8 [11-23]	93 [14-136]	7	70	7
Diplodus sargus	84	0	0	0	0	28.1 [22-38]	434.2 [204-968]	31	32	21
Engraulis encrasicholus	60	3.33±2.27	0.03±0.18	1±0	0.87	16.5 [13-18]	38 [32-48]	2	58	0

Afterwards, all the specimens were processed by the press method (Levsen andLunestad, 2010) and visually inspected under UV-light. To this end, fishes were gutted, manually skinned and thinly-sectioned (maximum 10 mm thick), and each fillet was introduced into a transparent resealable plastic bag and pressed to 2 mm thickness by means of a hydraulic press Mega 30 Ton KMG-30 (Melchor Gabilondo, S.A., Spain). After further frozen at -20°C for at least 12 hours, pressed fillets were visually inspected under an UV-light source in a Vilbert Lourmat CN-15.LC Cabinet (Vilbert Lourmat, Marne La Vallée, France) at 300-400 nm wavelength measure range and 365 nm excitation peak. Fluorescent images were captured with a camera Nikon D200 with lens AF-S Micro Nikkor 60 mm f/2.8G ED (Nikon Corporation, Tokyo-Japan). The picture of each entire sample was viewed at once for quick parasite counting, and any part of the image was enlarged for finer fluorescence resolution (even of shape and size) for parasite confirmation. From the total of 1910 fish specimens examined, only in doubtful cases the parasite counting was confirmed by carrying out the pepsin-HCl digestion procedure as a confirmatory golden method, based on Llarena-Reino et al. (2013a).

The terms prevalence (P), mean abundance (A), mean intensity (I), and density (D) of infection were determined following Bush et al. (1997) and Rozsa et al. (2000).

7.3. RESULTS

A large number of fluorescent images from each fish specimen were captured during the inspection in the cabinet, as a result of the visual scrutiny of pressed-frozen fish fillets under UV-light. After deleting distinguishable artefacts as spines, nerve tissue or remnants of skin in the fillets, any anisakid larvae present in the samples had the appearance of bright bluish-white spots or worms easily recognizable, against a darker background within the bags (Figure 7.1). All this diagnostic information collected has been incorporated into an image database with the aim of becoming part of a consultation network (Figure 7.2).

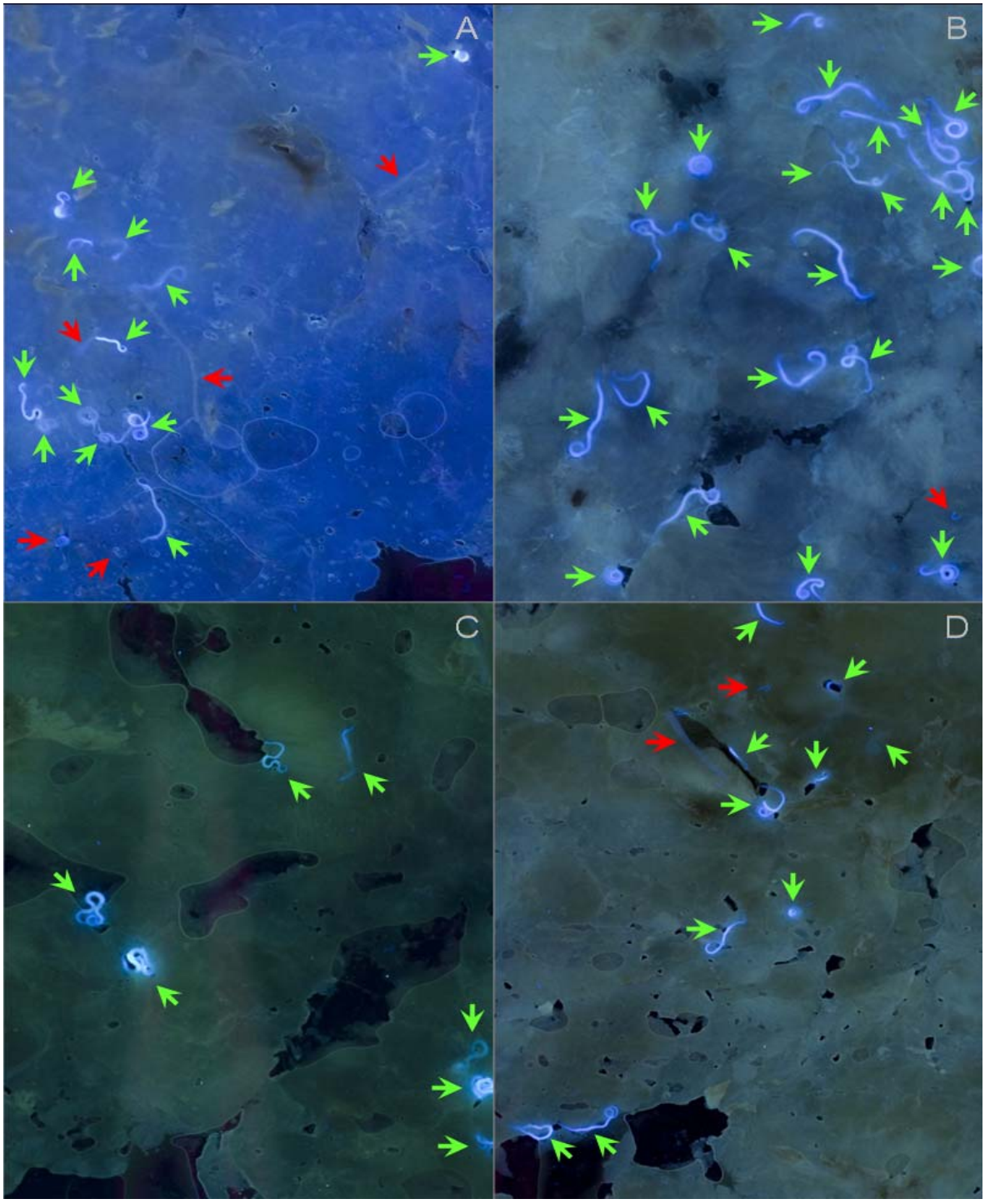
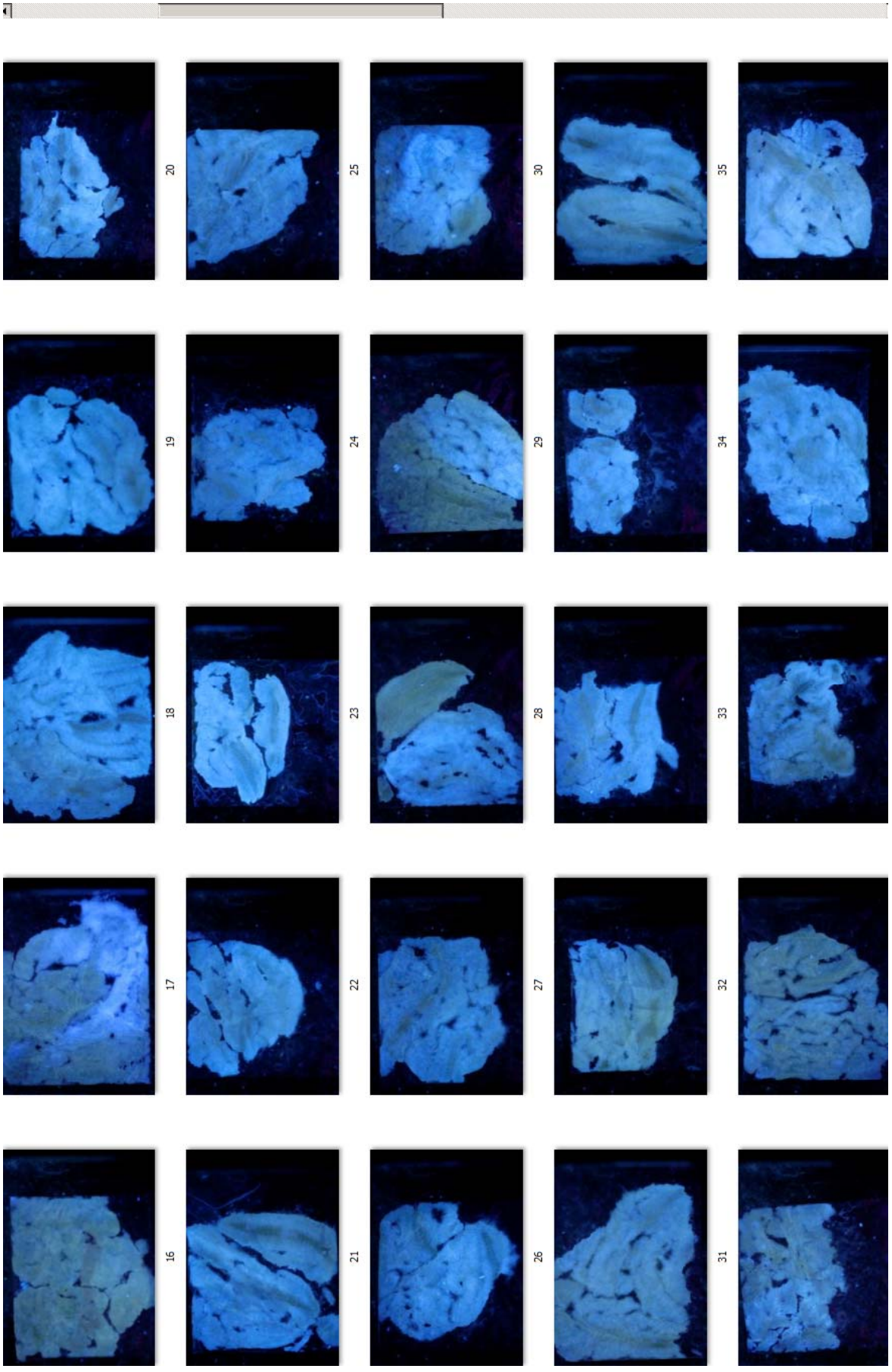


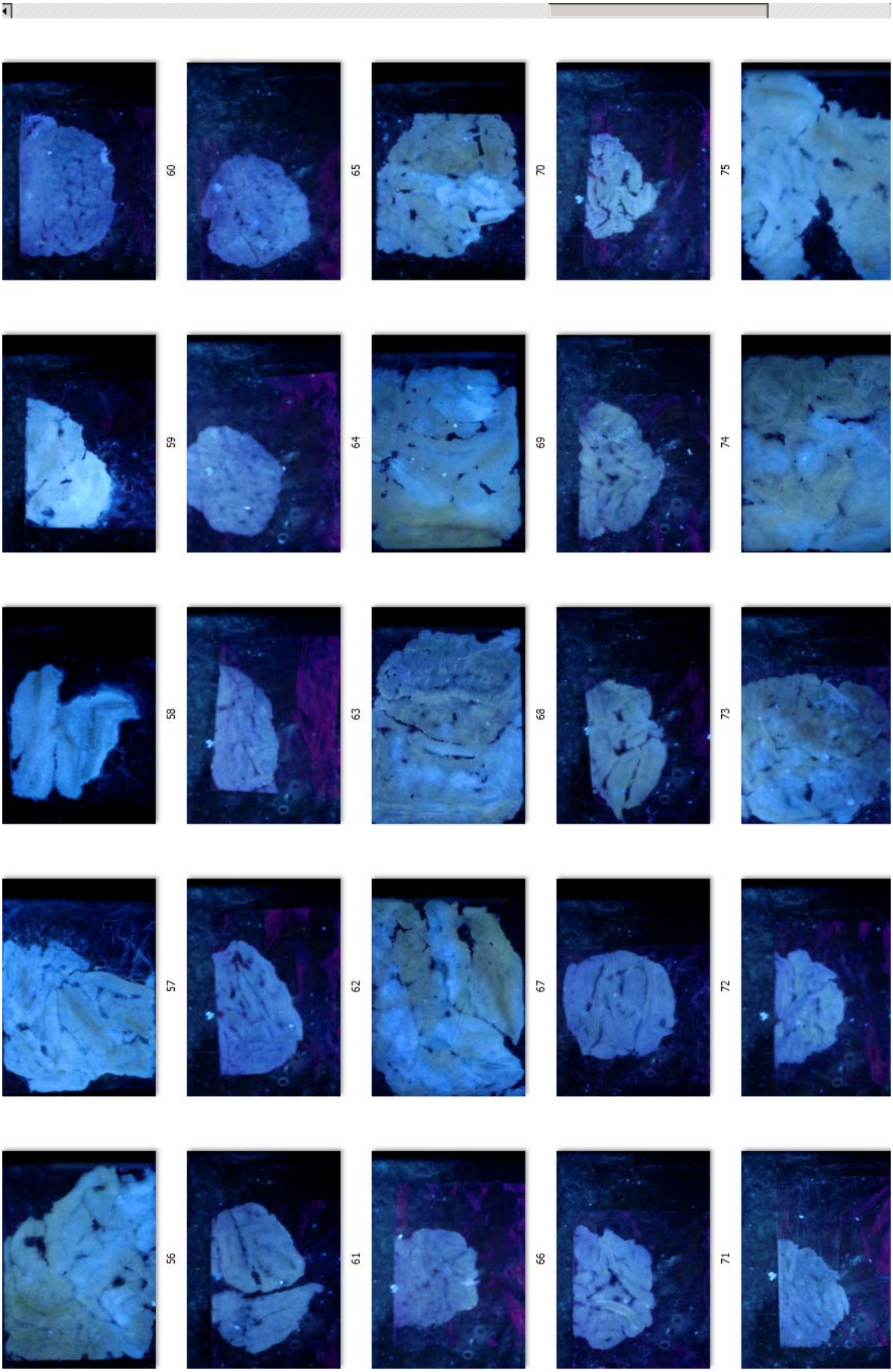
Figure 7.1. Images of *Merluccius merluccius* **(A, B)** and *Trachurus trachurus* **(C, D)** pressed fish fillets emitting auto-fluorescence under UV-light (365 nm of excitation), in a Vilbert Lourmat CN-15LC UV-Cabinet. Highly contrasting white spots/worms (green arrows) represent anisakid larvae within the fish muscle. Fluorescent artefacts are also shown (red arrows).

Figure 7.2. (A-M). Example of the image database generated in this study, after the inspection under UV conditions of pressed-frozen untrimmed fish fillets belonging to 25 lots. This extract illustrates fluorescent images from 13 fish lots/species. Anisakid larvae present appear in the pictures emerging as brightly auto-fluorescent spots. The straight lines mark the approximate boundary between the belly flaps and the dorsal musculature of each fish side.

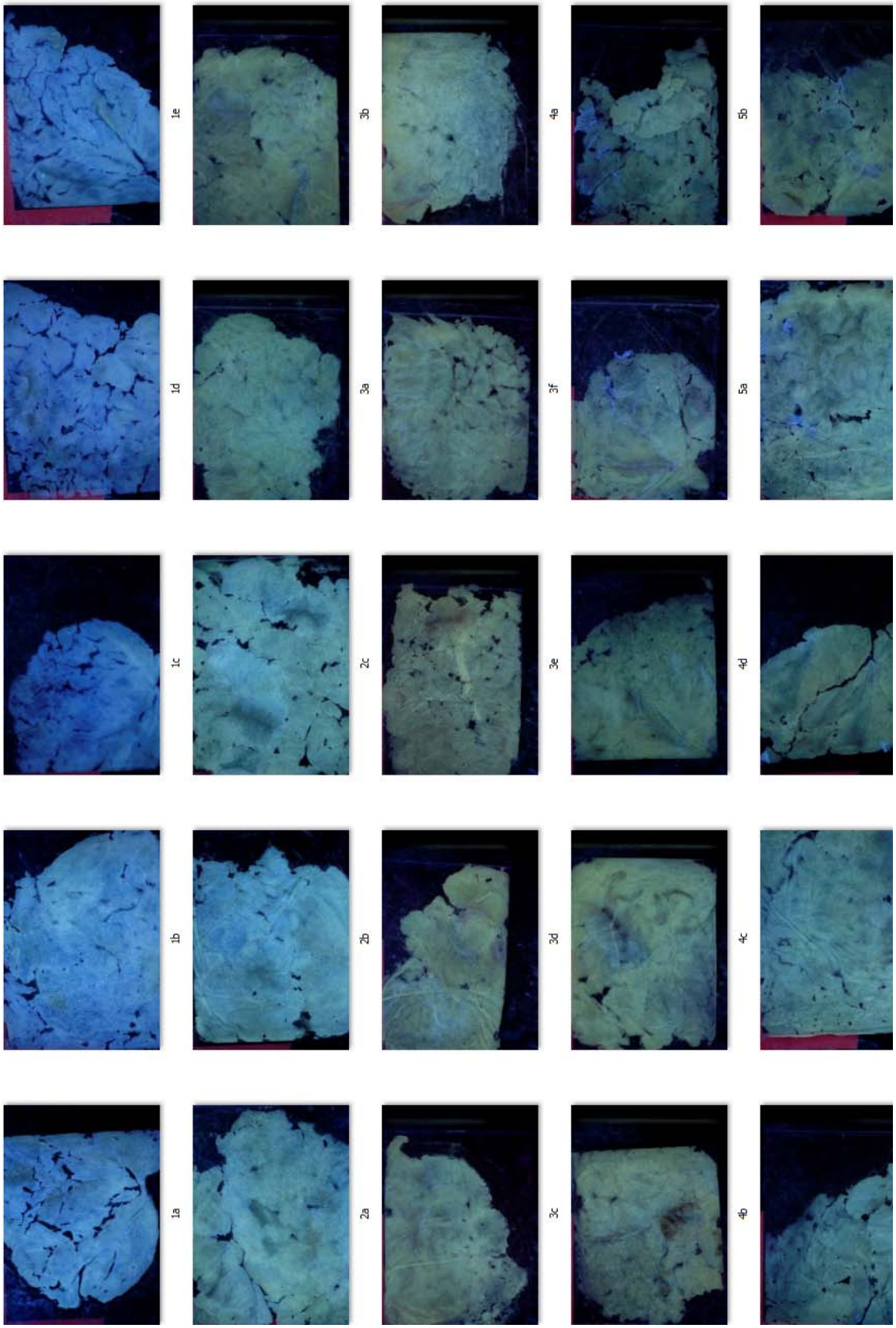
7.2.A. *Pegusa lascaris*



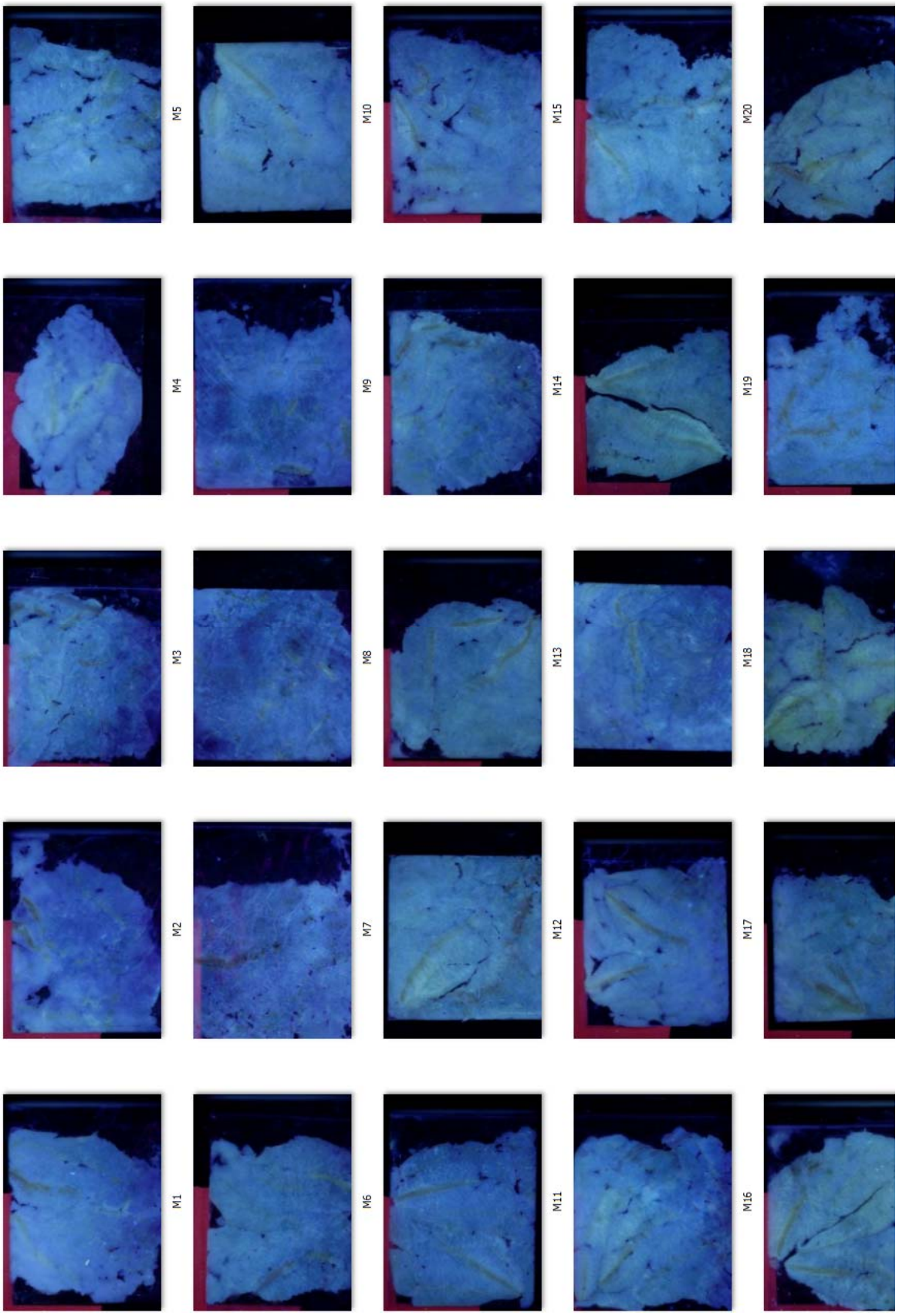
7.2.B. *Dicologlossa cuneata*



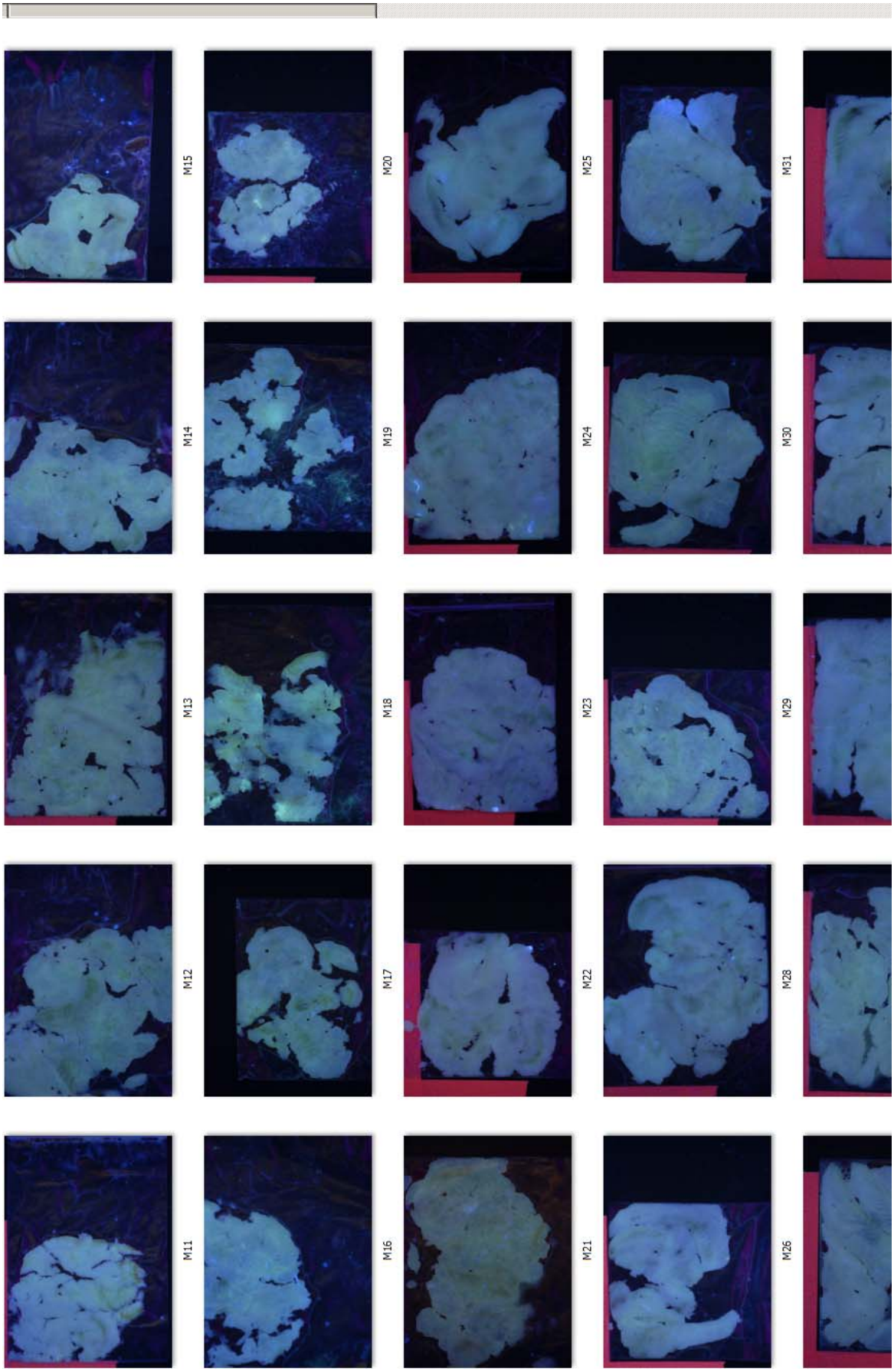
7.2.C. *Brama brama*



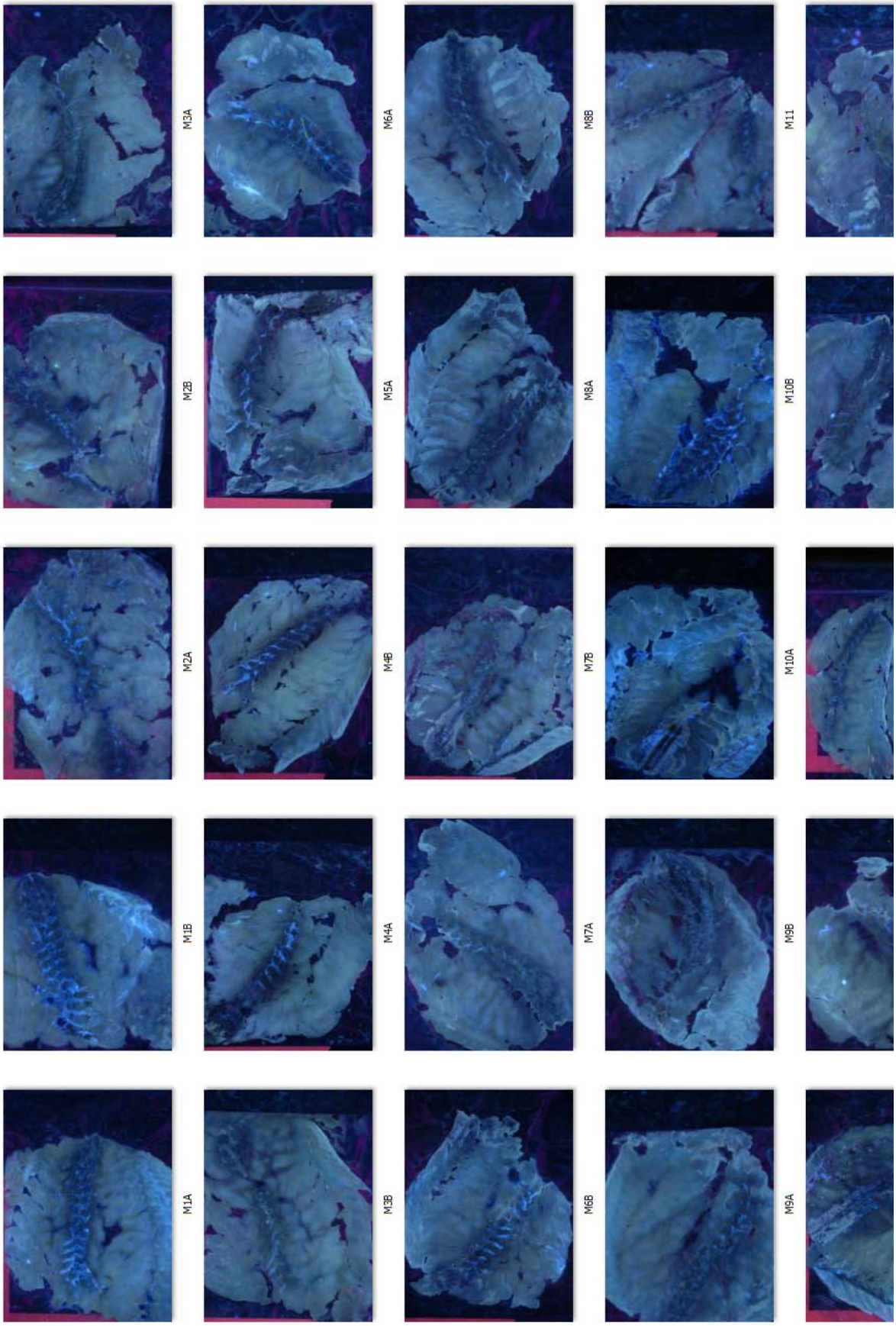
7.2.D. *Trisopterus luscus*



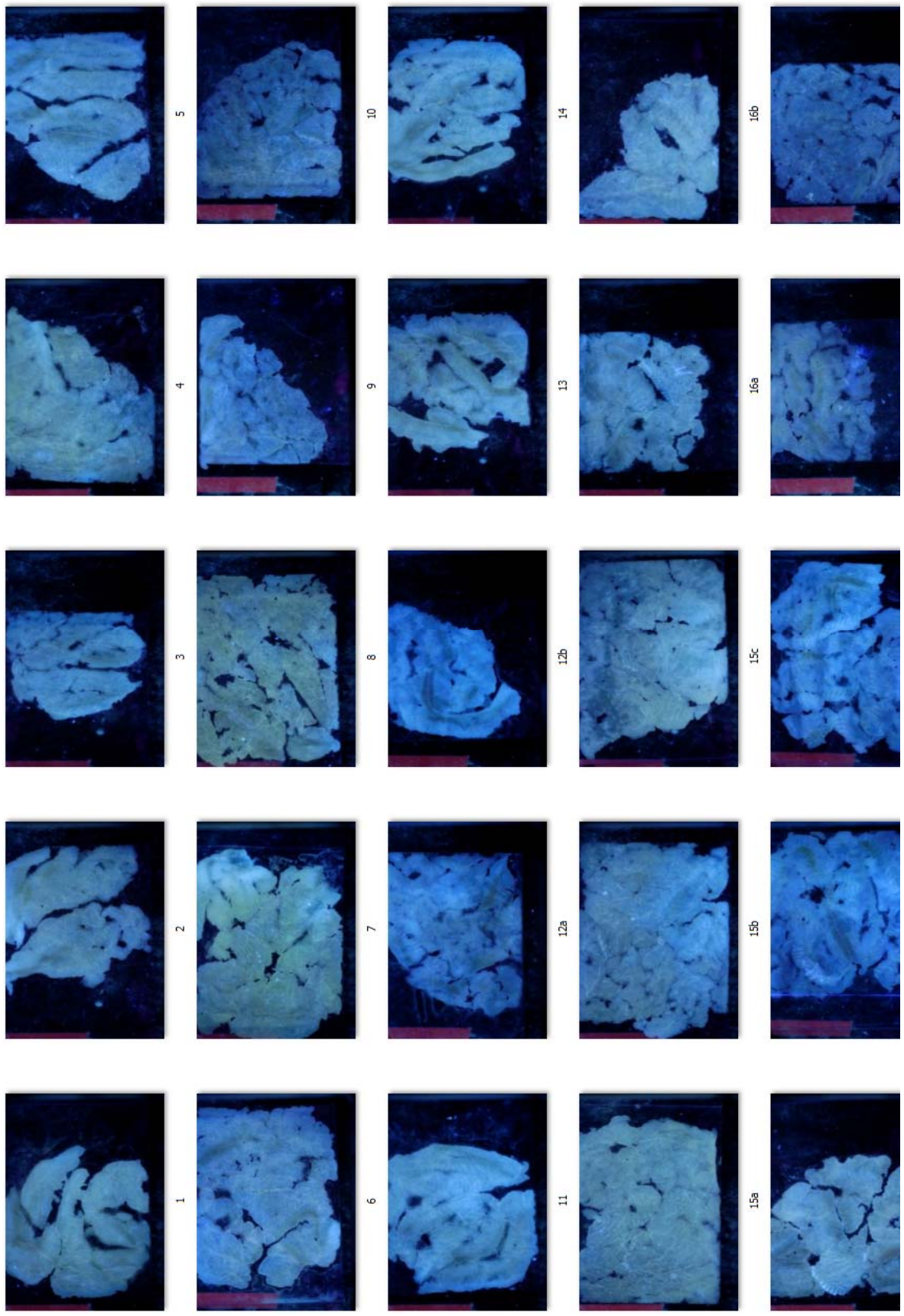
7.2.E. *Lepidorhombus* spp.



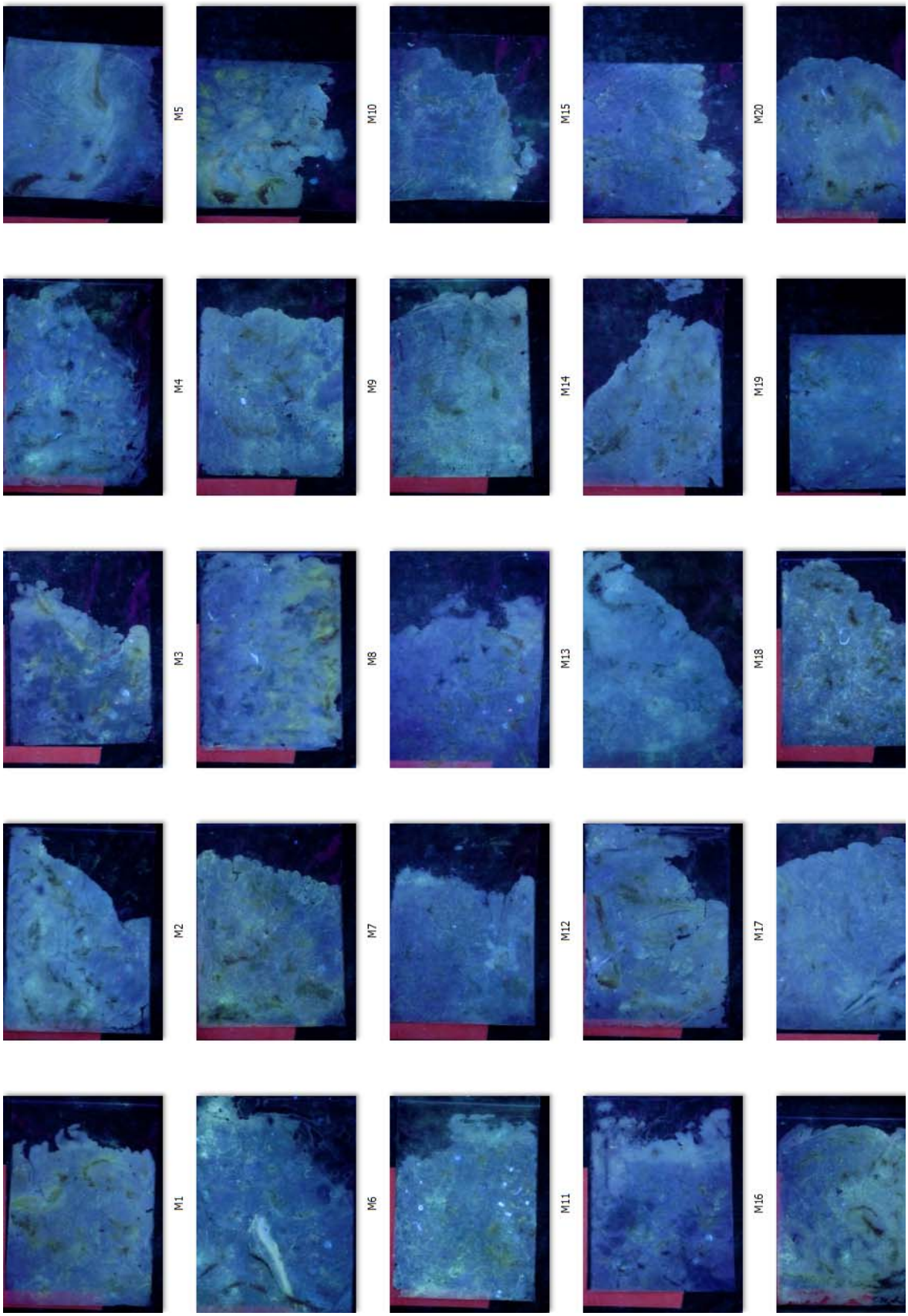
7.2.F. *Trachurus trachurus*



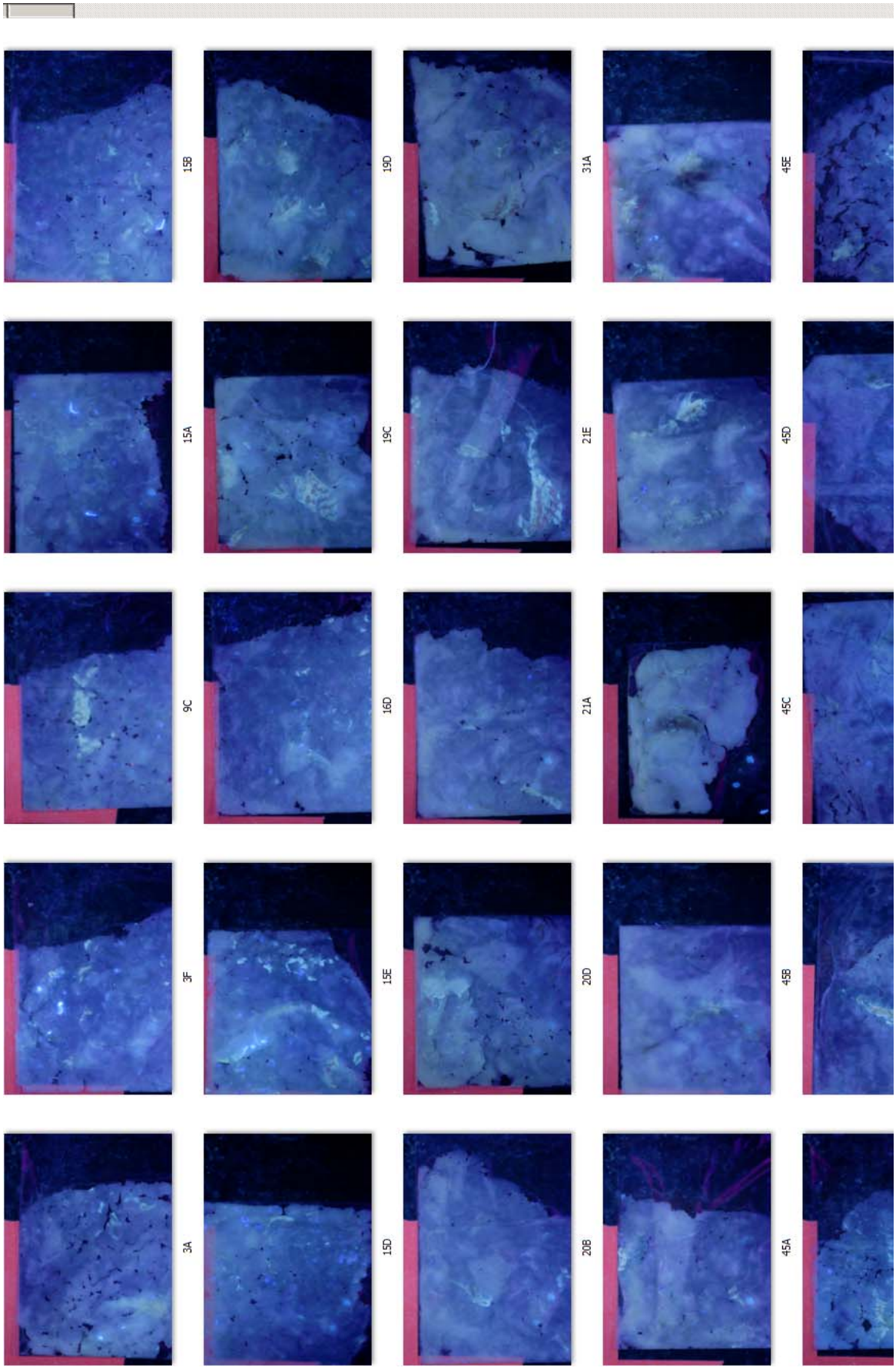
7.2.G. *Solea* spp.



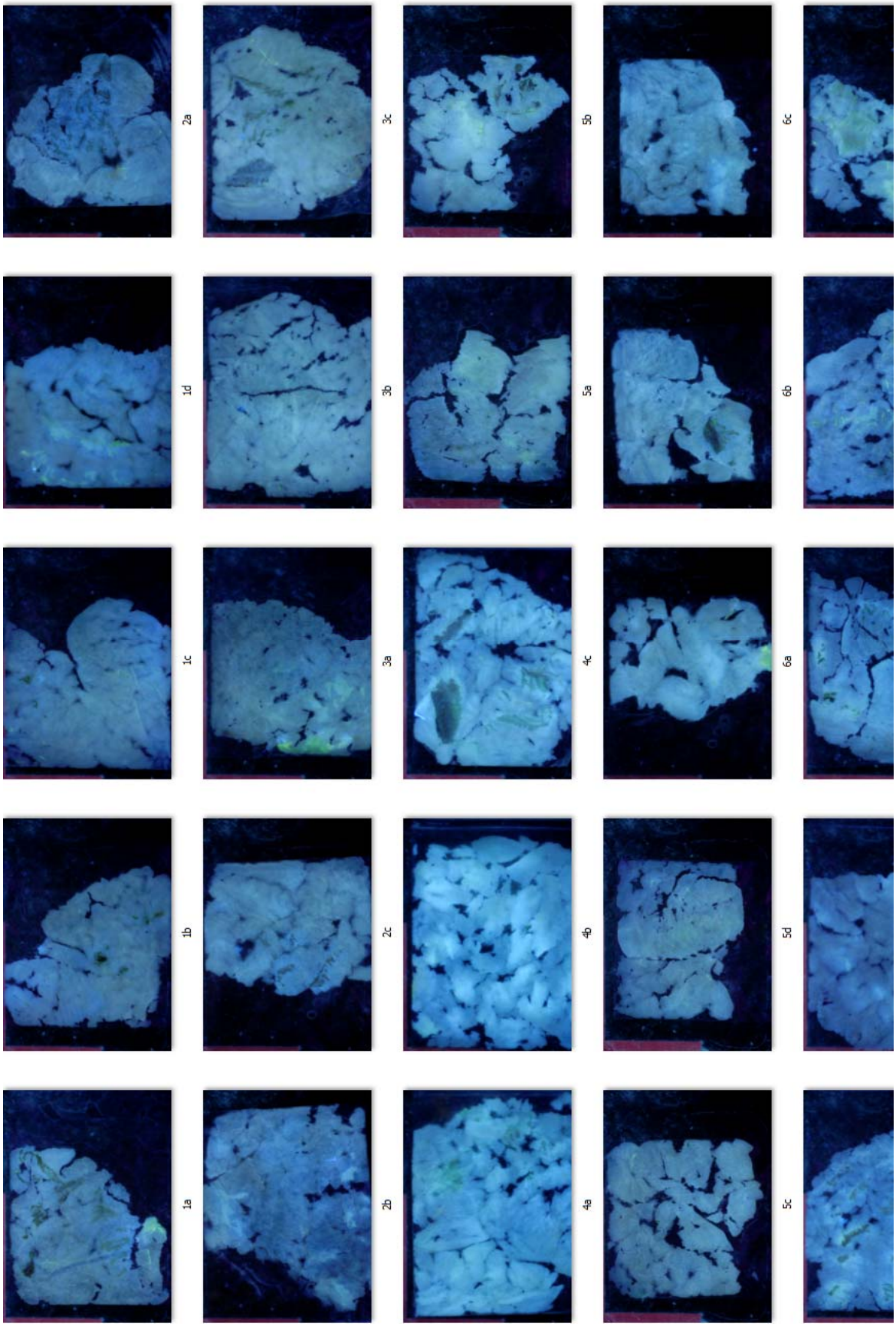
7.2.H. *Micromesistius poutassou*



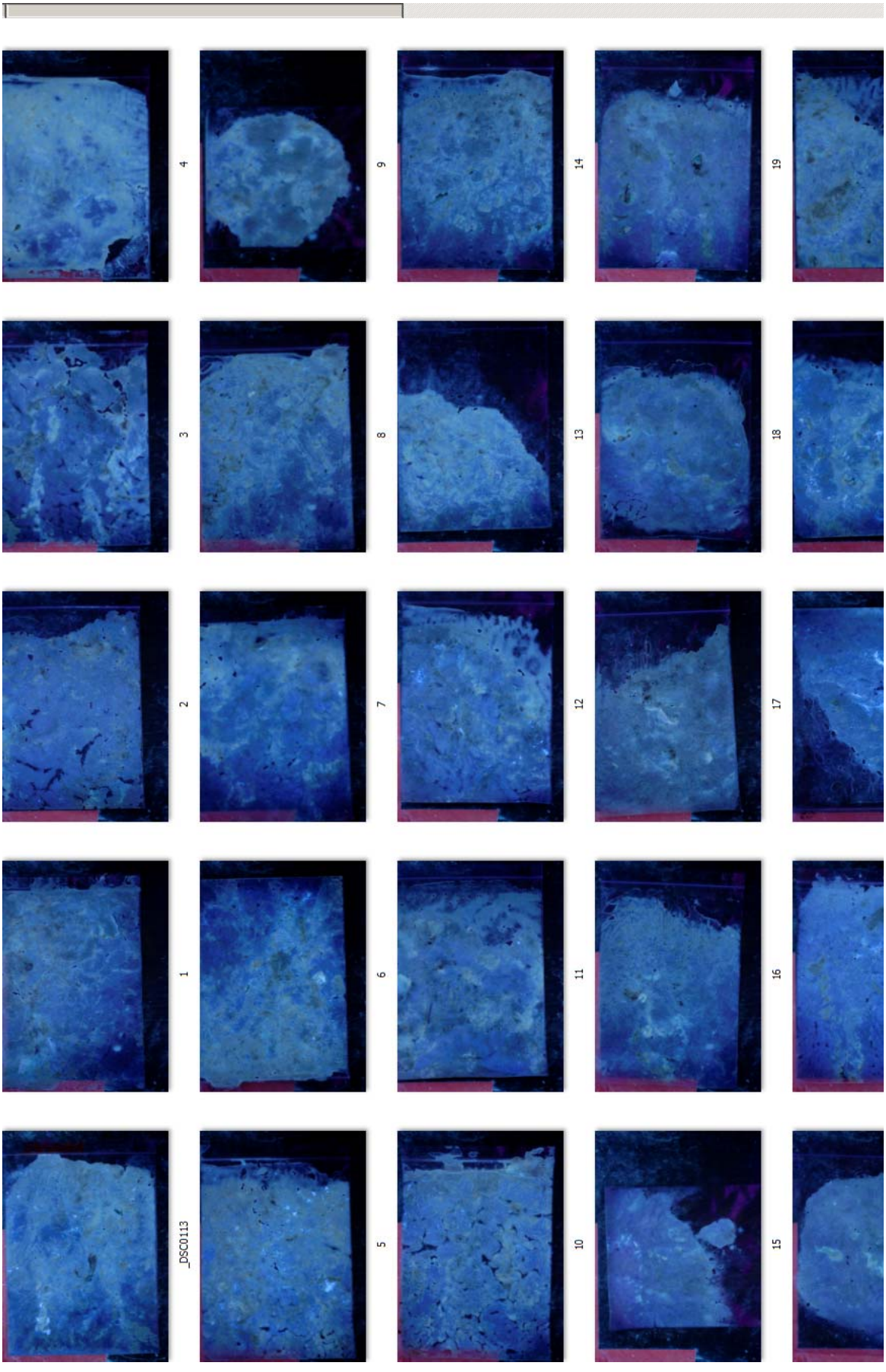
7.2.I. *Merluccius merluccius*



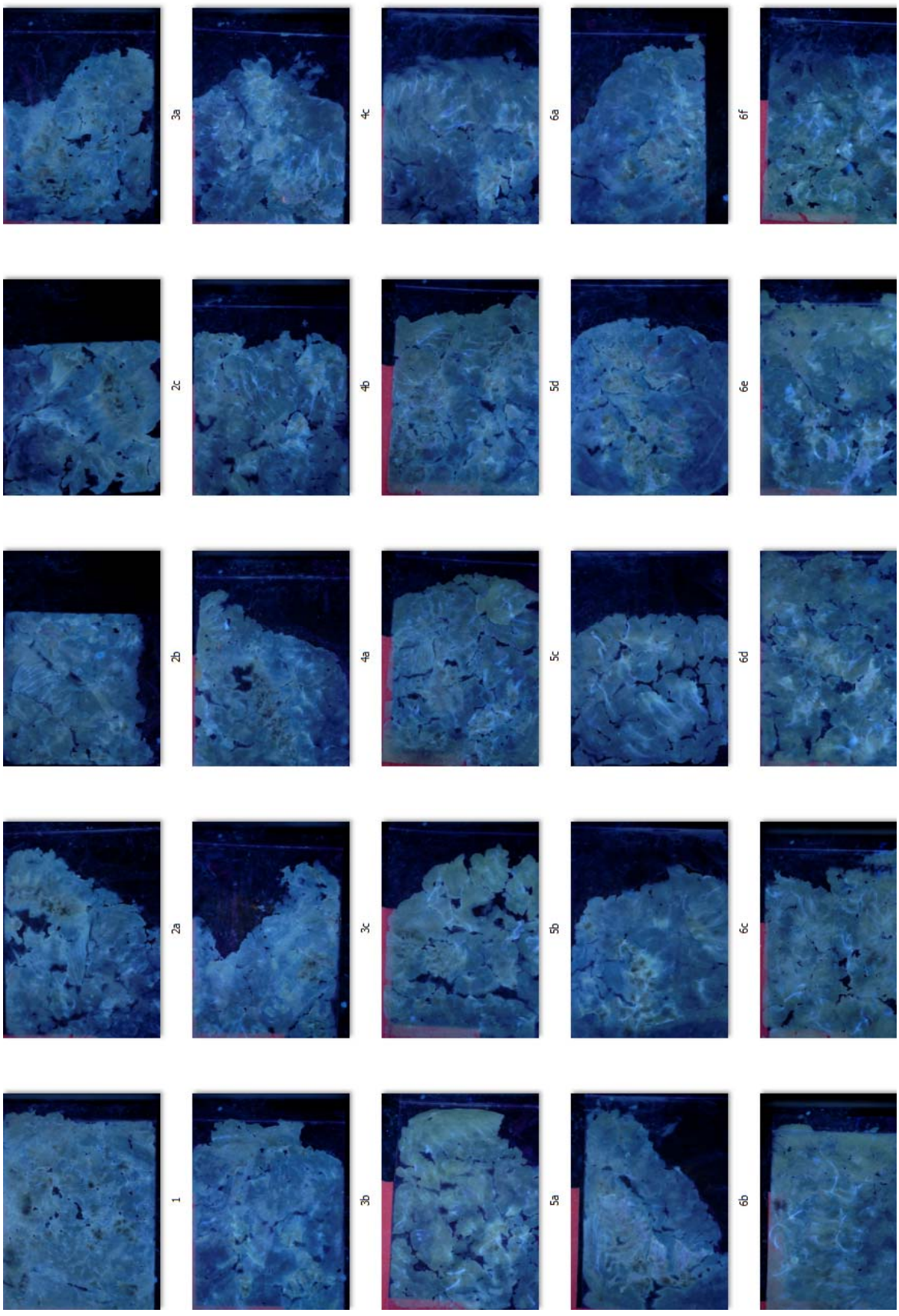
7.2.J. *Zeus faber*



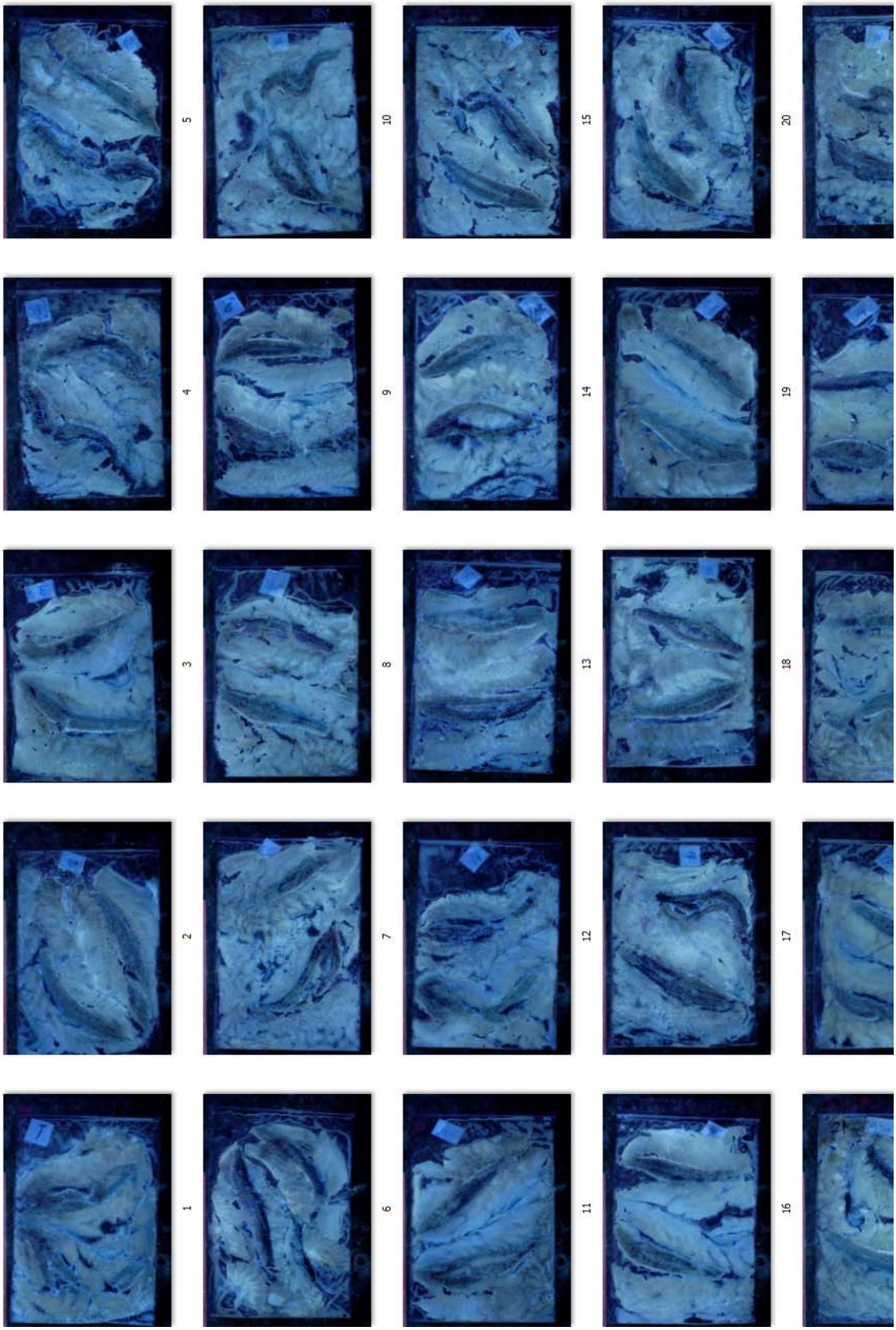
7.2.K. *Sardina pilchardus*



7.2.L. *Diplodus sargus*



7.2.M. *Engraulis encrasicholus*



In some cases during fish inspection by the press method, the existence of artefacts made difficult the differentiation of parasites. A total of 63 individuals created doubt about the absence/presence or concerning the number of anisakid larvae pre-diagnosed, so those specimens were submitted to a dual analysis. After reprocessing those samples by means of the pepsin-HCl digestion method, comparative results between both procedures were as shown in table 7.2.

Table 7.2. A comparative study between UV-Cabinet and pepsin-HCl digestion inspection procedures carried out in doubtful samples from each fish lot. The number of whole larvae found for each case is given. F: larval fragment. N: non anisakid larval.

Fish species	Test No.	Cabinet	Pepsin HCl	Fish species	Test No.	Cabinet	Pepsin HCl
Brama brama	1	1	1	*Solea* spp.	5	0	0
	2	0	0		6	0	0
	3	0	0		7	0	0
	4	0	0	*Micromesistius poutassou*	1	13	13
	5	0	0		2	2	2
Trisopterus luscus	1	0	0		3	18	18
	2	0	0		4	3	3
	3	0	0	*Merluccius merluccius*	1	6	6
	4	0	0		2	13	13
Lepidorhombus spp.	1	1	1		3	15	15
	2	0	0		4	19	19
	3	0	0		5	36	36
	4	1	1	*Zeus faber*	1	2	2
	5	2	2		2	1	1
	6	2	1+1F		3	2	2
	7	0	0		4	2	1+1F
	8	0	0		5	5	5
	9	0	0		6	6	5+1F
Trachurus trachurus	1	1	1		7	4	2+2F
	2	2	2		8	3	3
	3	2	2		9	1	1
	4	13	13		10	1	1
	5	1	1		11	0	1N
	6	1	1		12	1	1
	7	1	1		13	0	0
	8	1	1		14	2	2
	9	1	1		15	3	3
Solea spp.	1	0	0		16	2	2
	2	0	0		17	1	1
	3	0	0	*Sardina pilchardus*	1	1	1
	4	0	0		2	1	1

Only in four fishes; one individual of *Lepidorhombus* spp. and three belonging to *Zeus Faber*, five parasitic findings previously reported as “larvae”, were finally observed as “larvae fragments”. In conclusion, excepting for the case of test No. 11 in *Zeus Faber*, in which the nematode found was not an anisakid larval,

all the tests confirmed 100% detection efficiency by comparing parasite counts by the press method and parasite recoveries in re-examined samples after artificial digestion.

Demographic infection values presented in table 7.1 contained the highest densities of anisakid infection up to 13.34 and 4.54 larvae/kg in spring and autumn respectively for *M. poutassou,* and 5.42 (spring) and 18.22 larvae/kg (autumn) in the case of *M. merluccius*. Moreover, four of the twelve species captured in spring (*Lepidorhombus* spp., *T. trachurus*, *M. merluccius* and *Z. faber*) and only three from the thirteen species studied in autumn (*T. trachurus*, *M. poutassou* and *Z. faber)* were assessed as having secondary infection. Taking into consideration very low density values (<3) and levels of prevalence under 10%, it was concluded that the species *B. brama*, and *S. pilchardus* presented anisakids as accidental infection in spring, and *B. brama*, *T. luscus, Lepidorhombus* spp., and *E. encrasicholus* in autumn. Finally, five species during spring (*P. lascaris, D. cuneata, T. luscus, Solea* spp. and *D. sargus*) and five species during autumn captures (*P. lascaris, D. cuneata, Solea* spp., *S. pilchardus* and *D. sargus*), were free of muscular anisakids. With the exception of few cases, demographic values of infection did not differ significantly from one season to another. When comparing them, it can be observed that in autumn only one fish species (*T. luscus*) showed a slightly increase in its prevalence value, another five decreased significantly, and six of them maintained the same levels throughout the whole study. However, although four lots decreased their mean abundance and density values of anisakids infection in the second sampling period with regard to the first, other four species (*B. brama*, *T. luscus, M. merluccius* and *Z. faber*) evidenced some type of increase, which was particularly marked in the case of *M. merluccius.* A similar fact occurred in autumn over the previous season in relation to intensity levels in these four species, and also in *Lepidorhombus* spp.

7.4. DISCUSSION

Over 750000 tons per year of fresh fish commerce for human consumption has its origin in the Port of Vigo one of the most important fishing ports in the world. From a risk assessment point of view, an interesting conclusion can be drawn when compared our results with that of seroprevalence data for anisakids in this Galician area. As some recent scientific studies have demonstrated (Abollo et al., 2001; Llarena-Reino et al., 2013b), among other species, *M. merluccius* and *M. poutassou* from Vigo markets host anisakids. Moreover, apart from the contaminated flesh, the liver, gonads and viscera of all parasitized fish species have also showed great intensities of *Anisakis* infection; ≥58.9% of total worm burden (Abollo et al., 2001), which has already been recorded as an important source of anisakiosis and associated allergic disorders (Jurado-Palomo et al., 2010). Despite both fish species are important components of the diet of fish-consuming population in Galicia, low allergic hypersensitivity values for *Anisakis* spp. have been recorded in this specific area. This fact outlines that local traditional cooking preparations (which avoid raw or undercooked fish, light salted, pickled or smoked seafood) kill the nematodes and thus eliminate the possibility of human

infection priory required for possible allergic reactions, as stated in EFSA Scientific opinion (EFSA, 2010). However, the high national and international export activity carried out daily from the fishing Port of Vigo requires that strict *c*ontrol measures have to be adopted by the fish companies, through implementation of corrective actions in edible parts of heavily infected fish species as European hake and blue whiting. In addition, post-harvest prophylactic measures are an efficient control policy that should also be appropriately improved in national control programs. Otherwise, in a demonstrated European schema of fragile consumer perception it is important to avoid misconceptions about the safety of eating infected fish flesh by guaranteeing that no visible parasites which affects the aesthetic quality of the product and/or which are being broadcasted as an emergent contaminant in food alerts, reach the consumer.

The present findings and previous studies (Banning and Becker, 1978; Davey, 1972; Karl, 2008; Levsen et al., 2005) state that temporal and geographical variations in *Anisakis* spp. larval occurrence exists, presumably due to ecological and/or behavioral reasons. The present work provides the first data per seasons of the anisakid larvae presence in commercial fish species sold at retail level. With the exception of few cases, demographic values of infection did not differ significantly from one season to another. However, these variations could be due, among others, to environmental factors, variation in fishing grounds, change in fish feeding behaviors, intensity of fishing activity, or simply they could have been determined by chance. Slight parasitic variations between fishing grounds or species have to be considered for future studies as well as the creation of risk maps and related diagnostic tools that should be included in HACCP programs.

It seems remarkable to highlight the fact that the state of freshness of fishes with higher density values (*M. merluccius* and *M. poutassou*) was suboptimal for most of those individuals. It is difficult to demonstrate the potential inter-relationship existing between this fact and the possibility of migration of anisakids from gut to the flesh before evisceration, as previous authors have suggested (Llarena-Reino et al., 2012). In this regard, many factors could explain the possibility and timing (*intra-vitam* or *post-mortem*) of these migrations, mostly related to physiological trade-off of parasites, to ecological and immunological factors operating in living fish, or to the biochemical *post-mortem* changes which occur in autolyzed fish (Karl, 2008). Therefore, and taking into account the Scientific Opinion on risk assessment of parasites in fishery products (EFSA, 2010), which stated that "based on scientific evidences it is not clear when, under what conditions and in which fish species, *post-mortem* migration of *A. simplex* larvae occurs", future studies should be developed in further detail.

Finally, the dual analyzes performed in 63 from the total 1910 cases studied confirmed the consistency of the press-UV method. The reliability, work speed, user-friendliness, low investment cost, effectiveness, and diagnostic specificity which characterize this detection procedure have become it in an ideal candidate to be implemented as official standard method during self-control programs within the fishing sector. Moreover, the description of the fluorescent emission pattern and the basic principles of auto-fluorescence

of anisakids larvae, studied in Chapter 5, constitute a further step towards the development of a definitive industrial tool based on this method.

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CHAPTER 8

Inspection (III)

Case study: Copepods

Morphological and genetic identification of *Pennella instructa* (Copepoda: Pennellidae) on Atlantic swordfish (*Xiphias gladius*, L. 1758)

ABSTRACT

During the last years the presence of parasites in commercial fish species has been increasing at determined fisheries becoming an emergent major public health problem. For seafood companies the control of this biological hazard is turning into a priority issue, as quality of the products is now being seriously compromised. The swordfish *Xiphias gladius,* as one of the most important commercial species among European markets, has been inspected for the presence of pennellids. A total of 214 mesoparasitic copepods were sampled from 167 hosts in the fish auction market of Vigo (Spain), for epidemiology and genetic analysis. Moreover, 5 pennellid cephalothoraxes' were provided by a seafood processing company for morphological and genetic studies. Finally, a total of 50 slices of swordfishes parasitized with pennellids were supplied by another fishing company for examining the internal lesions and carrying out diagnostics on the basis of morphological characters. All hosts were captured between 2011 and 2012 in two NE Atlantic fishing areas, FAO 27 and FAO 34, specifically in waters comprising Macaronesia and areas close to Portuguese and Spanish coasts. Morphological and genetic results suggested that populations of *X. gladius* in the sampling area were infected by a pennellid species, *P. instructa*. The "parasite-host" anchorage scheme observed suggested that slices were also parasitized by this species, *P. instructa*. Morphological characters as well as internal and external lesions caused by this parasite were acutely described. Prevalence and mean Intensity of infection could be determined for the total fishes parasitized in the fish auction market. The prevention of rejections due to the presence of these parasites and cysts, and the damage they cause on organoleptic properties of fishes, must be the first step to ensure safer and high quality standard products to final consumers. Thus, monitoring actions and proactive self-inspections which include preventive and corrective measures should be more intensively integrated into HACCP systems of seafood companies.

KEYWORDS

Swordfish, *Xiphias gladius, Pennella instructa,* morphology, genetic

8.1. INTRODUCTION

The swordfish *Xiphias gladius,* is one of the most important species among European commercial fish stocks. This pelagic species, which extends from tropical to temperate-cold areas (Nakamura, 1985; Mattiucci et al., 2005, Garcia et al., 2011), is usually caught in Macaronesia, involving the whole area between Cape Verde and Azores. The waters close to Portugal and Spain are probably the most frequented fishing areas during the autumn months. On the contrary, March and April are the months when the fishing vessels increase their captures far from those coasts.

Despite the considerable economical importance that characterises the fishing ports of Portugal as Peniche, Matosinhos, Povoa de Varzim, Sesimbra or Algarve, many Portuguese fishing vessels dedicating their activity to swordfish often unload the fish catches in the fishing port of Vigo (Spain). Among other reasons, this is due to the fact that the swordfish is the 10th species of fresh fish with highest landings in that port, as the Fishing Statistical Information of the 2012 Annual Report from the Port Authority of Vigo states. Vigo's port is the most important fishing harbour of Spain and one of the most relevant ports in Europe in terms of landings. In addition, the commercial interest that *X. gladius* has on Spanish fishing sector and among consumers is higher than the Portuguese existing one, possibly due to cultural and gastronomic reasons.

Many members of the mesoparasitic family Pennellidae, order Siphonostomatoida, are characterised by needing intermediate hosts in their life cycle (Kabata, 1979; Abaunza et al., 2001). Closely related to swordfishes, the genus *Pennella*, one of the least known in its family due in part to the difficulty in obtaining individuals for description and to their phenotypic plasticity (Abaunza et al., 2001), probably constitute the most significant threat for this commercial fish species since it is becoming an emergent major public health problem. The great concern that this parasite is causing among companies within the fishing sector is making a further strengthening of the control of this biological hazard, as quality of the products is being seriously compromised.

The aim of this work was to determine the infection levels of the pennellid specimens that are infecting the swordfish population in five main fishing areas of Eastern Atlantic waters, their distribution by anatomical regions in the host body surface, and their morphological and genetic identification. This attempt to increase our knowledge about this problem intends to help develop effective solutions in the very short term and gives the fishing sector the chance to plan proactive measures and put them into operation, in order to minimize the high economic losses caused by rejections due to the presence of this parasite.

8.2. MATERIALS AND METHODS

8.2.1. Host inspection and parasite collection

A total number of 1631 swordfish individuals caught in the NE Atlantic fishing areas FAO 27 and 34, comprising the whole Macaronesia, from Cape Verde to Azores and the waters close to Portugal and Spain, were externally examined for the presence of pennellid copepods. Hosts' inspections took place in the fish auction market of the fishing port of Vigo (Spain) during 17 sampling days coinciding with the days of higher tonnage of landed fish, between March and September 2011. Throughout that 6-months period, a total of 15 Portuguese and 5 Spanish long line vessels were the main swordfish providers in that port. Hosts' body surface, natural orifices, gills, and fins were externally inspected for pennellids. All the external parasitic portions found (including trunk, abdomen with brush and egg strings), were measured and collected

without any kind of manipulation of hosts' bodies due to fish auction market's regulations. Parasite specimens were then adequately preserved in 70% ethanol until genetic studies. All data concerning capture information and biological characterization of the parasitized host were registered and are summarized in Table 8.1. Lower jaw furcal length (LJFL) and fork length (FL), and the round weight (RWT) were measured and recorded respectively from all parasitized swordfishes. In addition, information such as sampling date, port of origin of vessels, hosts fishing area and subarea and length of parasitic external portions, is also provided.

As Regulation (EC) 2074/2005 states in Section 1 of Annex II, a representative number of fishes has to be routinely submitted to a visual inspection by qualified technicians, at establishments on land and on board factory vessels. Persons responsible of that kind of measures must determine the scale and frequency of inspections depending on the type of the fish products, their geographical origin, and the final use they are intended for. Accordingly, some pennellid cephalothoraxes were found by the staff responsible for the quality control of a Spanish fish processing and trade company during routine inspections, evisceration and filleting processes. After subtraction from swordfish body tissues, these parasitic structures were immediately frozen at -20°C until dissection procedures, morphological description and identification. Subsequently, they were preserved in 70% ethanol for posterior genetic studies. Cephalothoraxes sampling was carried out between May and July 2012, and a restricted amount of detailed information about biological data of hosts and catches is provided, in order to safeguard the confidential information of the company.

Additionally, fifty parasitized slices belonging to six commercial swordfishes provided by a Spanish fish processing and distribution company were examined after thawing for the presence of pennellid copepods. Hosts were caught in May 2012 in fishing grounds from NE Atlantic fishing areas FAO 27 and 34. Slices were photographed and cysts were carefully measured in maximum wide and length. Parasitic fragments present in the slices were preserved in 70% ethanol and then processed for genetic studies. Uncontrolled freezing and thawing processes, as well as the filleting practice, made it impossible to consider those pennellid portions for morphological studies.

Most cephalothoraxes examined and all the parasitic fractions collected from slices were incomplete or fragmented due to eviscerating or filleting routine procedures. This circumstance coupled with the fact that the pennellid external portions removal was carried out without opening hosts, made it impossible to obtain the total length of any parasite.

Taking into account the specific geographical origin of hosts, five sampling grounds were defined and assigned to a large proportion of the total parasitized fishes, in order to have a more organized representation of the parasites collected (Figure 8.1).

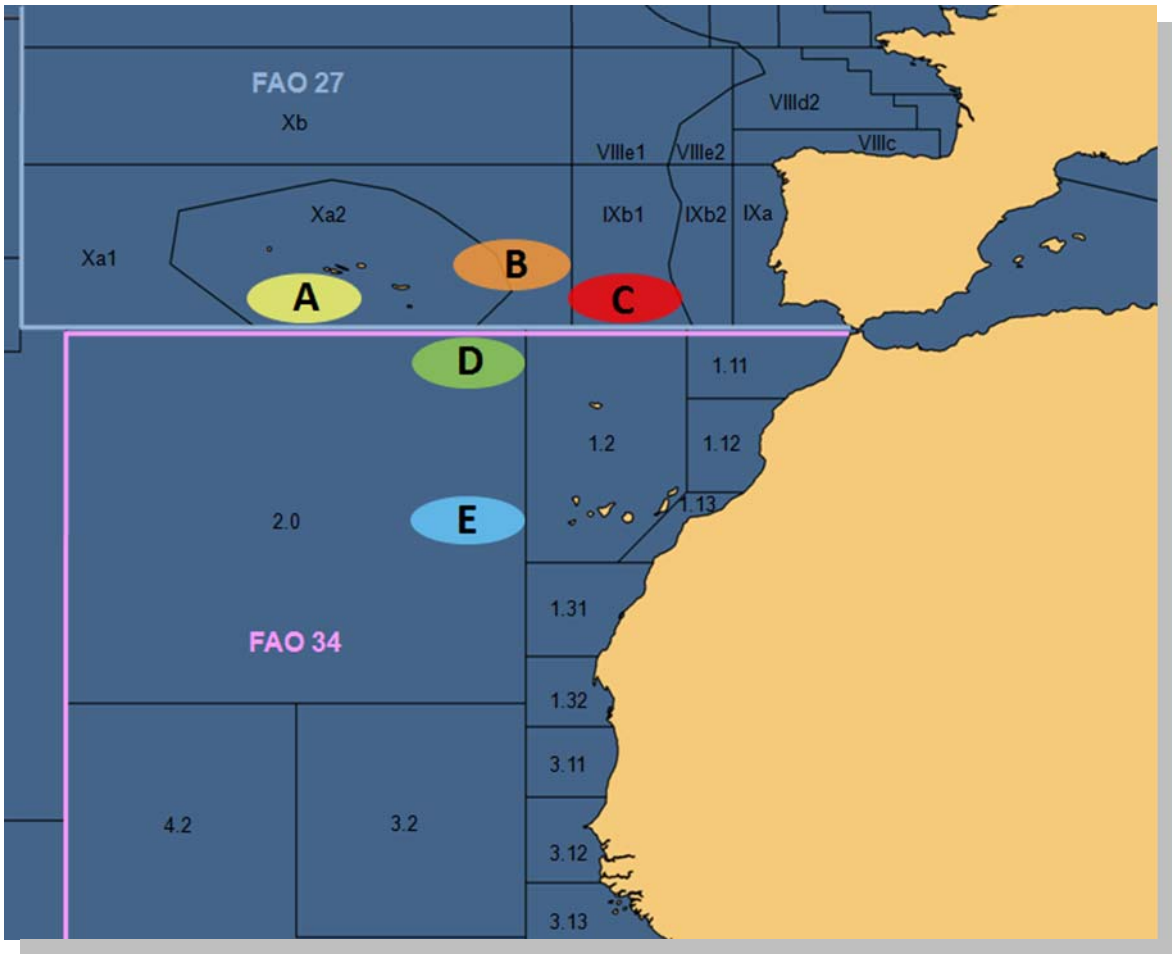

Figure 8.1. Map of partial NE Atlantic fishing areas FAO 27 and 34 with delimited subareas including the geographical origin of parasitized hosts. The pennellids genetically analyzed have been grouped by their origin in the five specific sampling grounds highlighted (A, B, C, D and E).

8.2.2. Morphological identification

For the morphological study of pennellids, each cyst provided by one of the seafood companies which collaborated was dissected after thawing, and cephalothoraxes were carefully isolated. These parasitic structures were submitted to a visual inspection with the aid of a Nikon SMZ800 stereomicroscope, and on the basis of their morpho-anatomical conformation, were identified following Hogans (1986). Several photographs of that process were taken for description and further taxonomic assignment.

8.2.3. Molecular identification

A total of 20 representative pennellid external portions (four from each fishing ground defined) and the 5 cephalothoraxes from the swordfish slices were prepared for molecular analysis. DNA extraction was carried out with NucleoSpin Tissue Kit (Macherey-Nagel, GmbH Düren, Germany) following the manufacturer's recommendations.

Targeted segments of the 18S rRNA gene were amplified using the primers 18SU467F (5'-ATCCAAGGAAGGCAGCAGGC-3') and 18SL1310R (5'-CTCCACCAACTAAGAACGGC-3') (Suzuki et al., 2006), and a partial segment of the 28S rRNA gene was amplified using the primers D9-D10-F (5'-CGGCGGGAGTAACTATGACTCTCTTAAGGT-3') and D9-D10-R (5'-CCGCCCCAGCCAAACTCCCCA-3') (Zardoya et al., 1995). All PCR mixtures were performed in a total volume of 25 µl containing 1 µl of genomic DNA (150-200 ng), PCR buffer at 1x concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science, Germany), 0.3 µM each primer and 0.025 $U.\mu l^{-1}$ Taq DNA polymerase (Roche Applied Science, Germany). The cycling protocol for the 18S rRNA gene was 2 min at 94°C, 35 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72°C, followed by 7 min at 72°C. The cycling protocol for the 28S rRNA gene was 2 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, followed by 7 min at 72°C. All PCRs were carried out in a TGradient thermocycler (Biometra GmbH, Goettingen, Germany) and a negative control (without DNA) was included for each set of PCRs. PCR products were separated on a 1.5% agarose gel in 1x TAE EDTA buffer, stained with 5 µl/100 mL RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea), and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories, Hercules CA, USA).

PCR products were purified for sequencing using Illustra ExoStar 1-STEP kit (GE Healthcare, UK Limited) according to the manufacturer's instructions. Sequencing was performed by the company Secugen S.L. (Madrid) using forward and reverse primers and the chromatograms were analysed using ChromasPro v.1.41 Technelysium Pty Ltd (South Brisbane, Australia). All generated sequences were searched for similarity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Centre for Biotechnology Information (USA).

Sequence sets for each gene (18S and 28S rRNA gene) were aligned in ClustalW multiple alignment of MEGA6 programme (Tamura et al., 2013) under default parameters. Alignments were used to construct phylogenetic trees using maximum likelihood (ML) and the best nucleotide substitution patterns for ML trees was selected based on the analyses of best-fit models in MEGA6. The ML trees were computed using the Jukes Cantor model of evolution with a bootstrap test (1000 replicates).

8.2.4. Demography of infection

The terms prevalence (P) and mean intensity (I) of infection were determined for the total of fishes parasitized from the fish auction market of Vigo, following Bush et al. (1997) and Rozsa et al. (2000). It was not possible to include pennellid cephalothoraxes neither slices in the estimations of those terms of infection, since the total number of fishes examined by both seafood companies is not known.

Although Table 8.1 reports the site of infection for each parasite collected in as much detailed as possible, for a better understanding and with the aim of illustrating more clearly the parasitized parts of the swordfish surface and their level of intensity, host body was divided into 10 anatomical regions (Figure 8.2).

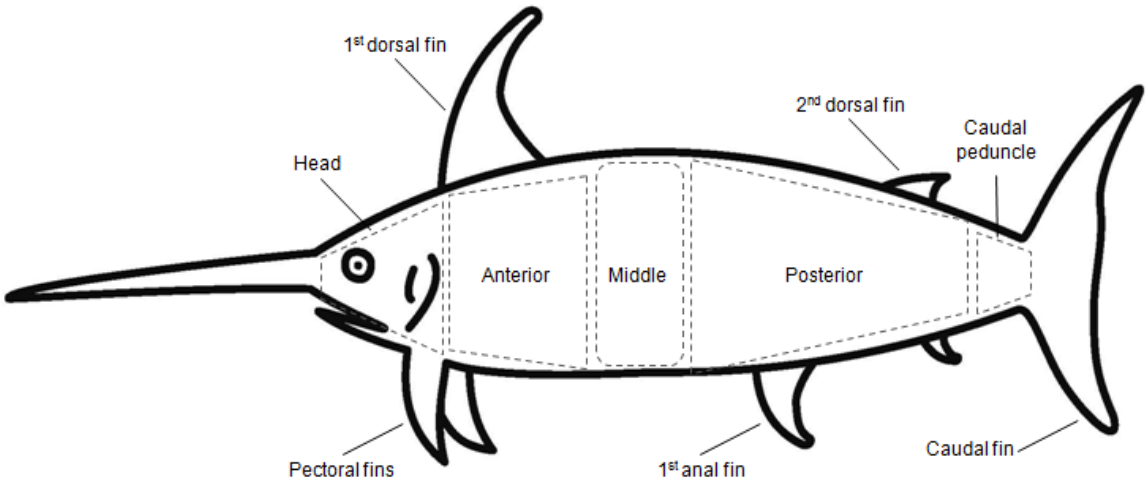

Figure 8.2. Swordfish body has been divided into ten anatomical regions, which may allow a better illustration of the degree of parasitic infection by zones of the examined hosts surface. Those regions comprise: anterior, middle and posterior sections, head, first and second dorsal fin, pectoral fins, first anal fin, caudal peduncle and caudal fin.

Specific terminology used in Table 8.1 to describe the location of pennellid external portions in hosts, includes some terms not contained in Figure 8.2, as dorsal, ventral or lateral, and the specific site of anchoring within the corresponding anatomic region.

8.3. RESULTS

8.3.1. Macroscopic examination

As Table 8.1 shows, lower jaw furcal length (LJFL) and fork length (FL) from parasitized fishes, varied from 97 to 283 cm and from 136 to 380 cm, respectively. Similarly, the round weight (RWT) presented a wide range from 9 to 257 Kg in the swordfishes sampled. These length and weight ranges from parasitized hosts were representative of the total individuals checked.

As result of a meticulous external inspection of swordfishes, a total of 214 outer pennellid portions (which included part of the neck, trunk, abdomen with brush, and egg strings) were registered and collected. Various type of damage directly related to the anchorage and presence of these parasites, as ulcerative injuries, internal cysts protruding externally, or wounded orifices in the skin, could be assessed during visual inspection and sampling (Figure 8.3).

Table 8.1. Pennellid external portions from the fish auction market, cephalothoraxes and pennellid fragments extracted from swordfish slices. The capture information about hosts containing date, area and subarea, and the fishing area code defined for each parasite, is given. Table also includes swordfish's biological data as lower jaw furcal length (LJFL), fork length (FL) and the round weight (RWT). Parasite anatomical location in host body, their external length, and gens analyzed where applicable have been detailed.

PARASITE CODE	HOST SPECIES	SAMPLING DATE	FISHING VESSEL'S PORT OF ORIGIN	HOST FISHING AREA	HOST FISHING SUBAREA	FISHING AREA CODE	LJFL (cm)	FL (cm)	RWT (Kg)	PARASITE LOCATION IN HOST BODY	PARASITE EXTERNAL LENGTH	18S	28S
1	*X. gladius*	08 March 2011	Burela (Galicia), Spain	FAO 27 (ICES)	X	Not assigned	238	332	174	Ventrolateral posterior half	3		
2	*X. gladius*	08 March 2011	Burela (Galicia), Spain	FAO 27 (ICES)	X	Not assigned	178	273	66	Ventrolateral posterior half	7		
3	*X. gladius*	09 March 2011	Galicia, Spain	FAO 27 (ICES)	X	Not assigned	144	205	34	Ventrolateral posterior half	5		
4	*X. gladius*	09 March 2011	Galicia, Spain	FAO 27 (ICES)	X	Not assigned	165	246	50	Ventrolateral posterior half	5		
5	*X. gladius*	29 March 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	125	185	18	Ventrolateral posterior half	9		
6	*X. gladius*	29 March 2011	Sagres, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	200	295	125	Ventrolateral posterior half	11		
7	*X. gladius*	29 March 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	178	262	85	Ventrolateral posterior half	4		
8	*X. gladius*	29 March 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	168	257	66	Ventral posterior half	10		
9	*X. gladius*	04 April 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	124	181	24	Ventrolateral posterior half	5		
10	*X. gladius*	04 April 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	129	189	27	Head (behind the lower jaw)	8		
11	*X. gladius*	04 April 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	185	276	85	Lateral posterior half	4		
12	*X. gladius*	12 April 2011	Galicia, Spain	FAO 34	2.0	E	168	244	64	Dorsal posterior half	8		
13	*X. gladius*	12 April 2011	Galicia, Spain	FAO 34	2.0	E	150	218	41	Ventral posterior half	10		
14	*X. gladius*	19 April 2011	Ponta Delgada (Azores), Portugal	FAO 27 (ICES)	99	Not assigned	238	337	150	Lateral posterior half	5		
15	*X. gladius*	19 April 2011	Ponta Delgada (Azores), Portugal	FAO 27 (ICES)	99	Not assigned	132	199	27	Lateral posterior half	4		

16	*X. gladius*	25 April 2011	Truck from port of Peniche, Portugal	FAO 34	3.2 (Cape Verde)	Not assigned	103	155	15	Ventral anterior half between pectoral fins	10			
17	*X. gladius*	25 April 2011	Truck port of Peniche, Portugal	FAO 34	3.2 (Cape Verde)	Not assigned	103	155	15	Ventral anterior half between pectoral fins	10			
18	*X. gladius*	25 April 2011	Truck from port of Peniche, Portugal	FAO 34	3.2 (Cape Verde)	Not assigned	103	155	15	Ventral anterior half between pectoral fins	8			
19	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	134	201	27	Lateral posterior half	11			
20	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	143	210	39	Lateral posterior half	6			
21	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	188	269	85	Dorsal anterior half lateral to 1st dorsal fin	4			
22	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	107	158	12	Dorsal middle section	3			
23	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	103	150	12	Pectoral fin insertion	3			
24	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	147	221	40	Ventral anterior half (next to another one)	5			
25	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	147	221	40	Ventral anterior half (next to another one)	5			
26	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	141	209	37	Lateral posterior half	3			
27	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	156	233	53	Lateral posterior half	5	X	X	
28	*X. gladius*	25 April 2011	Viana do Castelo, Portugal	FAO 34	NW Canaries	E	215	312	134	Head (under the lower jaw)	5			
29	*X. gladius*	25 April 2011	Vila Real S. Antonio, Portugal	FAO 34	3.2 (Cape Verde): 20°N 21°W	Not assigned	283	380	220	Lateral posterior half	2			
30	*X. gladius*	25 April 2011	Vila Real S. Antonio, Portugal	FAO 34	3.2 (Cape Verde): 20°N 21°W	Not assigned	157	236	51	Lateral posterior half	2			
31	*X. gladius*	25 April 2011	Vila Real S. Antonio, Portugal	FAO 34	3.2 (Cape Verde): 20°N 21°W	Not assigned	147	216	36	Lateral posterior half	8			
32	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	150	230	39	Lateral middle section	1			
33	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	191	281	88	Head (dorsal part of the gill cover)	4			
34	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	257	346	257	Dorsal posterior half	4			
35	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	257	346	257	Ventral anterior half between pectoral fins	5			

36	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	257	346	257	Ventral posterior half (anal opening)	6	X	X
37	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	257	346	257	Ventral posterior half (anal opening)	4		
38	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	172	262	82	Lateral posterior half	7		
39	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	118	175	20	Head (dorsal part of the gill cover)	6	X	X
40	*X. gladius*	29 April 2011	Sesimbra, Portugal	FAO 34	NW Canaries	E	127	188	22	Lateral posterior half	5		
41	*X. gladius*	02 May 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	D	102	148	14	Dorsal middle section	4		
42	*X. gladius*	02 May 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	D	102	153	11	Caudal peduncle	5		
43	*X. gladius*	02 May 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	D	118	180	22	A: Anterior to first anal fin insertion	6		
44	*X. gladius*	02 May 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	D	118	180	22	B: Anterior to first anal fin insertion	6		
45	*X. gladius*	02 May 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	D	118	180	22	C: Anterior to first anal fin insertion	6		
46	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	149	227	43	Lateral posterior half	5		
47	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	166	235	55	Lateral posterior half	5		
48	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	190	270	88	Lateral posterior half	5		
49	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	174	254	62	Dorsolateral posterior half	8		
50	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	141	198	29	Caudal peduncle	7		
51	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	149	227	43	Lateral posterior half	6		
52	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	259	333	177	Ventral anterior half under the breastbone	4		
53	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	189	276	96	First dorsal fin insertion	6		
54	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	158	222	44	Lateral to first anal fin insertion	15		
55	*X. gladius*	02 May 2011	Funchal, Portugal	FAO 27 (ICES)	99	Not assigned	124	176	24	Head (dorsal part of the gill cover)	8		

56	*X. gladius*	02 May 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	140	215	36	Lateral to first anal fin insertion	13
57	*X. gladius*	02 May 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	162	233	48	Behind the first anal fin	12
58	*X. gladius*	02 May 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	119	175	16	Lateral posterior half	5
59	*X. gladius*	02 May 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	106	159	15	Dorsal middle section	10
60	*X. gladius*	02 May 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	133	190	30	Anterior to first anal fin insertion	9
61	*X. gladius*	02 May 2011	Galicia, Spain	FAO 27 (ICES)	114D2-115D1	Not assigned	133	190	30	Anterior to first anal fin insertion	9
62	*X. gladius*	12 May 2011	Burela (Galicia), Spain	FAO 34	35-38°N 37-44°W	Not assigned	130	181	19	Ventrolateral posterior half	13
63	*X. gladius*	12 May 2011	Burela (Galicia), Spain	FAO 34	35-38°N 37-44°W	Not assigned	131	190	25	Dorsal posterior half	15
64	*X. gladius*	12 May 2011	Burela (Galicia), Spain	FAO 34	35-38°N 37-44°W	Not assigned	164	209	29	Dorsolateral anterior half	4
65	*X. gladius*	23 May 2011	Vila Real S. Antonio, Portugal	FAO 27 (ICES)	IXa (38°35N/ 37°49N 10°15W)	Not assigned	204	300	103	Lateral anterior half	1
66	*X. gladius*	23 May 2011	Vila Real S. Antonio, Portugal	FAO 27 (ICES)	IXa (38°35N/ 37°49N 10°15W)	Not assigned	128	189	29	Lateral posterior half	4
67	*X. gladius*	23 May 2011	Vila Real S. Antonio, Portugal	FAO 27 (ICES)	IXa (38°35N/ 37°49N 10°15W)	Not assigned	128	189	29	Lateral posterior half	4
68	*X. gladius*	23 May 2011	Vila Real S. Antonio, Portugal	FAO 27 (ICES)	IXa (38°35N/ 37°49N 10°15W)	Not assigned	139	209	30	Caudal fin (dorsal surface)	12
69	*X. gladius*	23 May 2011	Vila Real S. Antonio, Portugal	FAO 27 (ICES)	IXa (38°35N/ 37°49N 10°15W)	Not assigned	134	203	35	Head (dorsal surface)	4
70	*X. gladius*	26 May 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXa (outside Berlenga I.)	Not assigned	129	189	25	Lateral posterior half	2
71	*X. gladius*	26 May 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXa (outside Berlenga I.)	Not assigned	97	136	9	Lateral to first anal fin insertion	10
72	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	131	197	29	Lateral middle section	6
73	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	211	315	167	Ventral middle section	13
74	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	211	315	167	Ventral posterior half	4
75	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	142	199	32	First anal fin	10

76	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	121	196	25	Ventral caudal peduncle	10				
77	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	111	163	16	Dorsal posterior half	8				
78	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	136	193	29	Ventral posterior half	12				
79	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	136	193	29	Dorsal posterior half	12		X	X	
80	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	107	153	13	Lateral posterior half	6				
81	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	129	196	25	Lateral posterior half	1				
82	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	135	198	20	Lateral middle section	4				
83	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	128	183	22	Left pectoral fin insertion	4				
84	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	127	182	27	Caudal fin (dorsal surface)	9				
85	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	120	180	17	Lateral posterior half	6				
86	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	103	149	11	Lateral middle section	2				
87	*X. gladius*	26 May 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2, (20°W 35°N)	D	150	235	55	Lateral middle section	4				
88	*X. gladius*	26 May 2011	Leixoes, Portugal	FAO 27 (ICES)	99	Not assigned	192	276	114	Ventral middle section	5				
89	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	171	254	75	Ventral posterior half	10				
90	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	171	254	75	Ventral posterior half	10		X	X	
91	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	155	235	50	Ventral middle section	4				
92	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	155	235	50	Ventral middle section	15				
93	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	143	204	35	Lateral posterior half	4				
94	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	126	182	26	Ventral posterior half	5				
95	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	130	195	28	Ventrolateral posterior half	9				

96	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	142	208	45	Dorsolateral posterior half	5				
97	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	144	216	52	Dorsal caudal peduncle	13				
98	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	200	293	101	Ventral middle section	4				
99	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	128	190	26	Ventral posterior half	4				
100	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	135	200	31	Ventral posterior half	15				
101	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	183	265	77	Ventral posterior half	10				
102	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	196	290	102	Lateral posterior half	5				
103	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	127	194	27	Ventrolateral posterior half	5				
104	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	133	198	32	Ventral posterior half	10				
105	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	150	220	39	Lateral posterior half	15				
106	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	146	212	34	Ventral posterior half	3				
107	*X. gladius*	26 May 2011	Sesimbra, Portugal	FAO 34	34 2.0 COPACE (21°N 28°W)	E	137	203	35	Dorsal posterior half	10				
108	*X. gladius*	09 August 2011	Vigo (Galicia), Spain	FAO 27 (ICES)/FAO 34	NW Canaries	E	201	292	113	Lateral posterior half	1 (cut)				
109	*X. gladius*	09 August 2011	Vigo (Galicia), Spain	FAO 27 (ICES)/FAO 34	NW Canaries	D	201	292	113	Ventral posterior half	4	X	X		
110	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	190	276	99	Ventral posterior half (anal opening)	1				
111	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	159	244	55	Lateral posterior half	3	X	X		
112	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	169	252	57	Ventral posterior half	2 (cut)				
113	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	142	199	36	Lateral posterior half	4				
114	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	142	199	36	Lateral posterior half	4				
115	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	142	199	36	Lateral posterior half	5				

116	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	150	223	40	Lateral posterior half	3			
117	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	150	223	40	Lateral posterior half	1			
118	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	150	223	40	Lateral posterior half	10			
119	*X. gladius*	18 August 2011	Vila do Conde, Portugal	FAO 27 (ICES)	COPACE 34.2 Xa2 (20°W 35°N)	D	150	223	40	Lateral posterior half	3	X	X	
120	*X. gladius*	18 August 2011	Funchal, Portugal	FAO 27 (ICES)	38°N 21°W (Xa1-Xa2	A	136	203	29	Lateral posterior half	2			
121	*X. gladius*	18 August 2011	Funchal, Portugal	FAO 27 (ICES)	38°N 21°W (Xa1-Xa2)	A	143	203	29	Left pectoral fin insertion	8			
122	*X. gladius*	18 August 2011	Funchal, Portugal	FAO 27 (ICES)	38°N 21°W (Xa1-Xa2)	A	139	206	37	Ventral posterior half	4			
123	*X. gladius*	18 August 2011	Funchal, Portugal	FAO 27 (ICES)	38°N 21°W (Xa1-Xa2)	A	130	192	26	Lateral posterior half	1 (cut)			
124	*X. gladius*	18 August 2011	Funchal, Portugal	FAO 27 (ICES)	38°N 21°W (Xa1-Xa2)	A	136	203	29	First anal fin insertion	2			
125	*X. gladius*	18 August 2011	Funchal, Portugal	FAO 27 (ICES)	38°N 21°W (Xa1-Xa2)	A	147	219	40	Ventral posterior half (anal opening)	4			
126	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	123	171	23	Ventrolateral posterior half	5			
127	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	123	171	23	Ventrolateral posterior half	5			
128	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	123	171	23	Ventrolateral posterior half	5			
129	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	123	171	23	Ventrolateral posterior half	5			
130	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	117	170	16	First anal fin insertion	14			
131	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	116	163	17	Lateral posterior half	12	X	X	
132	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	180	256	76	Ventral posterior half	5			
133	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	108	153	13	First anal fin insertion	4			
134	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	144	202	44	First anal fin insertion	1	X	X	
135	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	154	224	46	Lateral posterior half	6			

136	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	154	224	46	First anal fin insertion	1			
137	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	154	224	46	Dorsal posterior half	1			
138	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	238	340	181	Lateral posterior half	1			
139	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	238	340	181	Lateral posterior half	4			
140	*X. gladius*	18 August 2011	Âncora, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	238	340	181	Lateral posterior half	4			
141	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	160	237	46	First dorsal fin insertion	3			
142	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	146	220	42	First anal fin insertion	3			
143	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	142	220	33	Ventral middle section	7			
144	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	145	210	33	Ventral middle section	15			
145	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	186	281	79	Ventral middle section	10			
146	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	224	323	136	Dorsal posterior half	8			
147	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	224	323	136	Lateral posterior half	8			
148	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	127	195	25	Lateral posterior half	6			
149	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	135	205	32	Caudal peduncle	6			
150	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	146	220	42	Dorsal posterior half	5	X	X	
151	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	146	220	42	Dorsal posterior half	5			
152	*X. gladius*	22 August 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	205	282	108	Lateral middle section	8			
153	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	168	245	61	Pectoral fin insertion	2			
154	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	224	323	136	Dorsal middle section	6			
155	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	135	201	32	Lateral posterior half	1			

156	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	229	331	167	First anal fin	4				
157	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	229	331	167	Caudal fin (dorsal surface)	4				
158	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	229	331	167	Pectoral fin insertion	4				
159	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	164	244	58	First anal fin insertion	4				
160	*X. gladius*	22 August 2011	Vila Praia de Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb1, (40°N 15-20°W)	B	207	292	116	Lateral middle section	6				
161	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	149	220	38	First anal fin	9		X	X	
162	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	127	192	23	Head (behind the gill cover)	3				
163	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	121	170	18	First dorsal fin insertion	4				
164	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	121	170	18	Second dorsal fin insertion	4				
165	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	121	170	18	Lateral posterior half	1				
166	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	121	176	19	Caudal peduncle	5				
167	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	121	176	19	First anal fin	3				
168	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	121	176	19	Caudal fin (dorsal surface)	10				
169	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	146	211	42	Dorsal posterior half	11		X	X	
170	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	113	164	17	Lateral posterior half	4				
171	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	113	158	17	First anal fin	10				
172	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	118	173	17	Caudal peduncle	4				
173	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	150	220	39	Caudal peduncle	4				
174	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	109	157	16	First dorsal fin	8		X	X	
175	*X. gladius*	22 August 2011	Âncora, Portugal	FAO 27 (ICES)	Xa2-IXb (East of Azores)	B	131	198	27	Head (gill cover)	5				

176	*X. gladius*	29 August 2011	Sagres, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	124	208	28	Caudal fin	15	X	X
177	*X. gladius*	29 August 2011	Sagres, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	185	263	83	First anal fin insertion	5	X	X
178	*X. gladius*	29 August 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	140	206	39	Head (dorsally to eye)	11		
179	*X. gladius*	29 August 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	119	180	24	Lateral posterior half	6		
180	*X. gladius*	29 August 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	138	202	40	Head (behind the lower jaw)	6		
181	*X. gladius*	29 August 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)/FAO 34	Xa2(Azores)/ Canaries	A	124	176	22	First anal fin insertion	4		
182	*X. gladius*	08 Sept 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	IXb1 (41°N 17°W)	C	204	291	120	Ventrolateral middle section	4		
183	*X. gladius*	08 Sept 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	IXb1 (41°N 17°W)	C	204	291	120	Lateral posterior half	3		
184	*X. gladius*	08 Sept 2011	Viana do Castelo, Portugal	FAO 27 (ICES)	IXb1 (41°N 17°W)	C	138	213	32	Lateral posterior half	4		
185	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	153	219	50	Dorsal posterior half	13		
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187	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	109	160	15	Lateral posterior half	3		
188	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	180	261	89	First anal fin	6		
189	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	113	159	14	First anal fin	3		
190	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	148	220	43	First anal fin	8		
191	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	148	220	43	First anal fin	5		
192	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	148	220	43	First anal fin	4	X	X
193	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	112	160	15	Lateral middle section	4		
194	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	112	160	15	Lateral middle section	4		
195	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal	FAO 27 (ICES)	IXb1 (42°N 14°W)	C	114	168	14	Lateral middle section	3	X	X

196	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal		FAO 27 (ICES)	IXb1	(42°N 14°W)	C	114	168	14	Lateral middle section	4				
197	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal		FAO 27 (ICES)	IXb1	(42°N 14°W)	C	114	168	14	Lateral middle section	4				
198	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal		FAO 27 (ICES)	IXb1	(42°N 14°W)	C	202	276	111	Lateral posterior half	1				
199	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal		FAO 27 (ICES)	IXb1	(42°N 14°W)	C	202	276	111	Lateral middle section	5				
200	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal		FAO 27 (ICES)	IXb1	(42°N 14°W)	C	113	171	18	Lateral middle section	1				
201	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal		FAO 27 (ICES)	IXb1	(42°N 14°W)	C	113	171	18	Lateral middle section	3				
202	*X. gladius*	08 Sept 2011	Povoa de Varzim, Portugal		FAO 27 (ICES)	IXb1	(42°N 14°W)	C	113	171	18	Lateral middle section	3				
203	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	127	201	30	First anal fin	7				
204	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	127	201	30	Lateral middle section	2				
205	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	127	201	30	Lateral middle section	2		X	X	
206	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	127	201	30	Lateral middle section	4				
207	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	134	203	31	Dorsolateral posterior half	12		X	X	
208	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	134	203	31	First anal fin	8				
209	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	138	205	36	Dorsal posterior half	6				
210	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	126	198	28	Ventral posterior half	8				
211	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	156	232	58	Lateral middle section	8				
212	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	134	201	32	Ventrolateral middle section	8				
213	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	140	205	35	Lateral middle section	3				
214	*X. gladius*	08 Sept 2011	Vila do Conde, Portugal		FAO 27 (ICES)	IXb1	(38°N 17°W)	C	154	226	42	Head (behind the gill cover)	5				

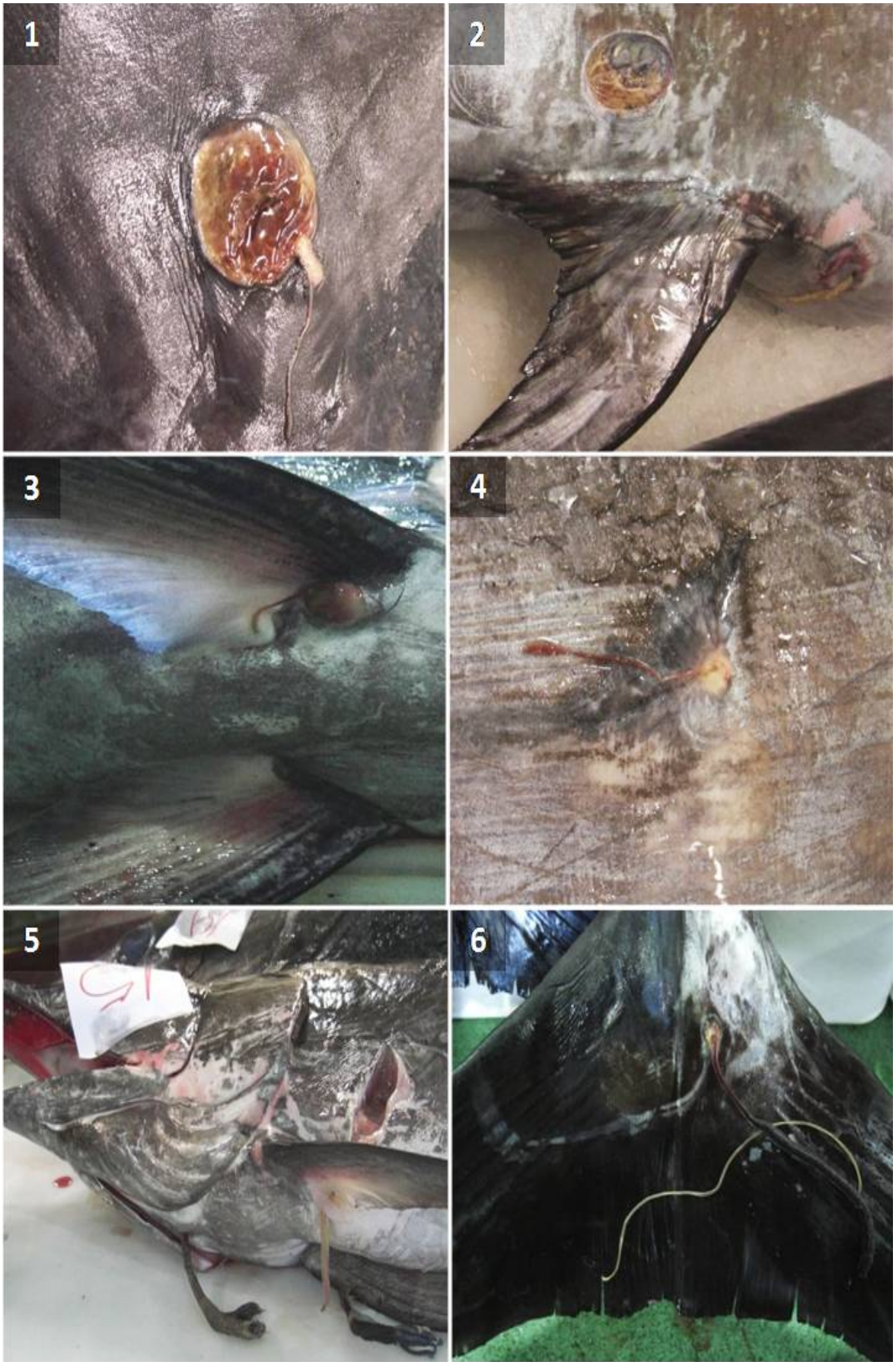
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Figure 8.3. (1-6). General and macro photographs of specimens of *Pennella instructa* anchored in diverse regions of *Xiphias gladius*. **1-2**: Pennellid external portion including part of the neck, trunk, and abdominal brush emerging from the lateral anterior half of the host body. An evident injury caused by the presence of this parasite is observed ulcerating fish external tissues. Picture 2 shows another skin ulcer, apparently without external pennellid fragment. **3**: Posterior fraction of a pennellid anchored in the right pectoral fin, forming an internal cyst which protrudes outward. **4**: Detail of the posterior end of a pennellid anchored in the surface of a host causing an external wound. **5**: External portion of two pennellid parasites showing part of the neck, the trunk, and abdominal brush emerging from the ventral face of the head, and from the right pectoral fin. **6**: Posterior end of a *Pennella* sp. penetrating the caudal fin tissues, and showing part of the neck, trunk, and abdominal brush. Some type of harm is observed in the surroundings of the anchorage point.

During slices inspection, numerous and apparent parasitic cystic forms, rounded, oval or elongated in shape, were observed within the host musculature (Figure 8.4). Their measures ranged from a minimum 1.5 cm (wide) to a maximum 5.1 cm (long). The specific anatomical location of cysts within each slice was apparently arbitrary; from dorsal to ventral regions, but in most cases rather deep in the musculature or medial areas, near vertebrae. In some cases it was possible to detect the presence of pennellid sections (cephalothorax portions including lateral horns and neck) inside the cysts.

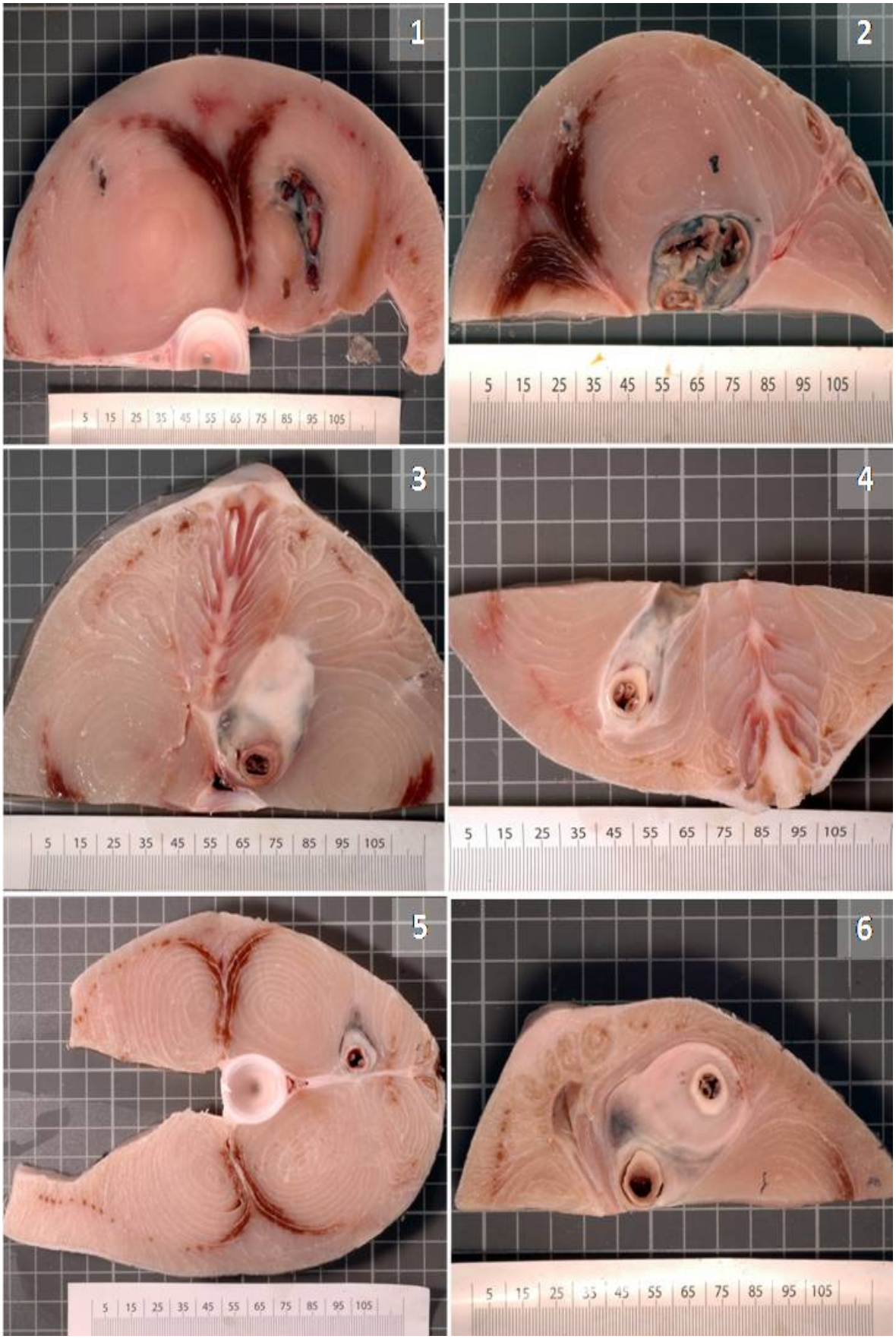
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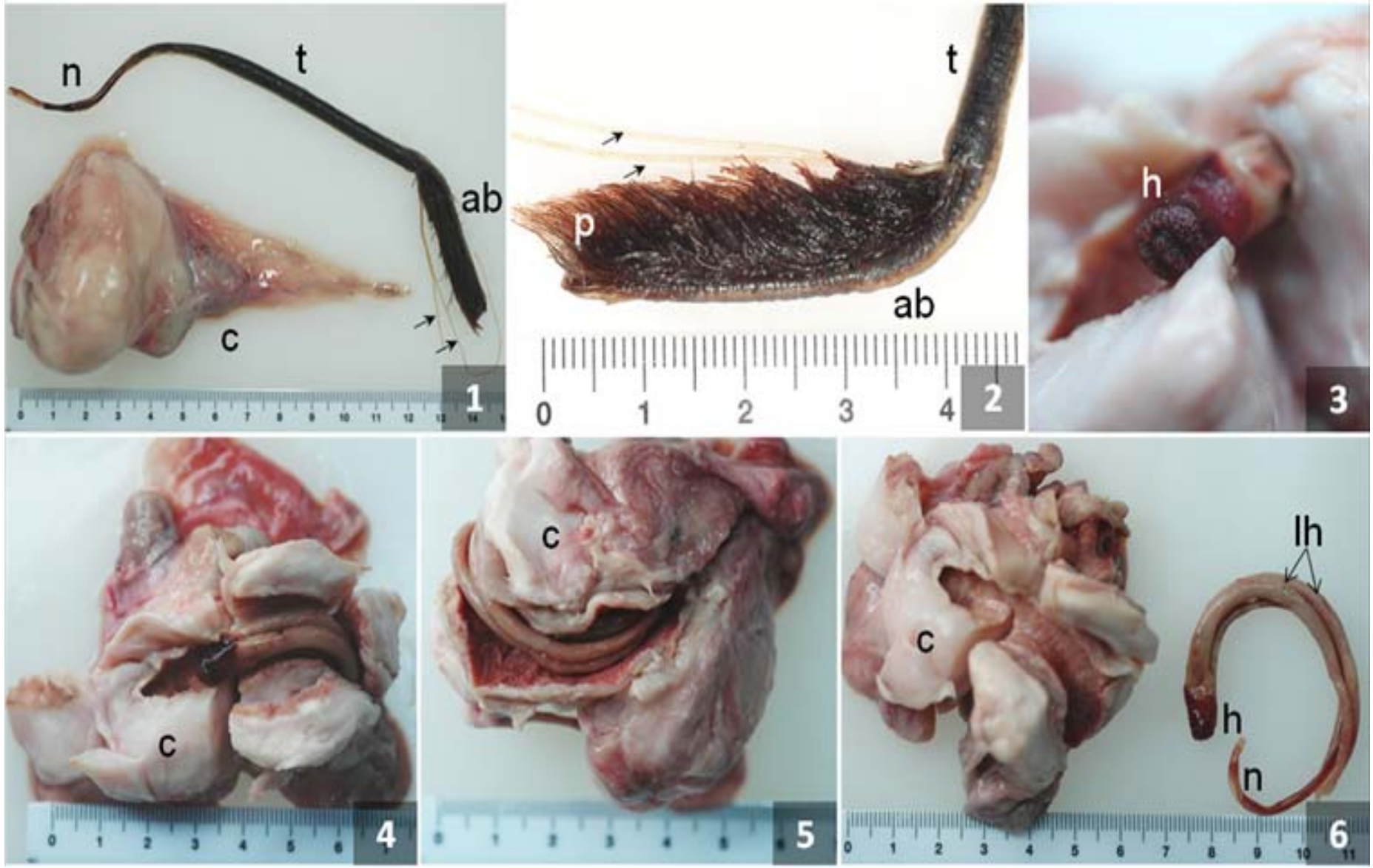
Figure 8.4. (1-6). A series of detailed pictures taken from slices of swordfish showing parasitized areas inside the musculature. **1**: Elongated cyst in the anterior section of the body, located in the middle of the musculature of the ventral region. Some parasitic zones are slightly visible. **2**: Rounded fibrous cyst in the posterior section of the body, located in the dorsomedial musculature. Some parasitic zones can be deduced. **3**: Oval cyst in the posterior section of the body, located in the ventromedial musculature. A thickened layer of connective tissue and a transversal section of the two lateral horns of the parasite can be observed. **4**: Elongated cyst in the posterior section of the body, located in the middle of the musculature of the ventral region. A thickened layer of connective tissue and a transversal section of the two lateral horns and the neck of the parasite can be observed. **5**: Rounded fibrous cyst in the anterior section of the body, located in the dorsomedial musculature. Two parasitic transversal sections are visible. **6**: Rounded cyst in the posterior section of the body, occupying the half musculature of the ventral region. A very thick layer of connective tissue and a transversal section of the two lateral horns and the neck of the parasite are evident.

8.3.2. Morphological identification

Cephalothoraxes together with a small portion of neck were found in all cases covered with a thick host tissue layer conforming apparent parasitic cysts, very large ones, at times. After thawing and during the dissection and visual inspection processes, each cephalothorax was carefully separated from the host tissue. The five pennellids targeted in the morphological study showed similar conformation and characteristics of anatomical structures (Figure 8.5). Each one presented a cylindrical cephalothorax with a flattened anterior end coated with two central and two lateral rows of small papillae, also known as antennary processes. Between the two central rows of antennary processes, a trilobed structure was distinguished arising, hiding the buccal complex. In all the cephalothoraxes two single papillae were also observed just below the central rows of the head, on the upper neck's ventral face. Moreover, two unbranched lateral horns were found in all specimens emerging from the head and extending backwards parallel to the neck. No adjacent or smaller bifurcations in the lateral horns were observed. The lengths in centimeters, measured from head's anterior end to posterior tips of lateral horns were: 8.7, 4.8, 11, 4.9 and 3.8.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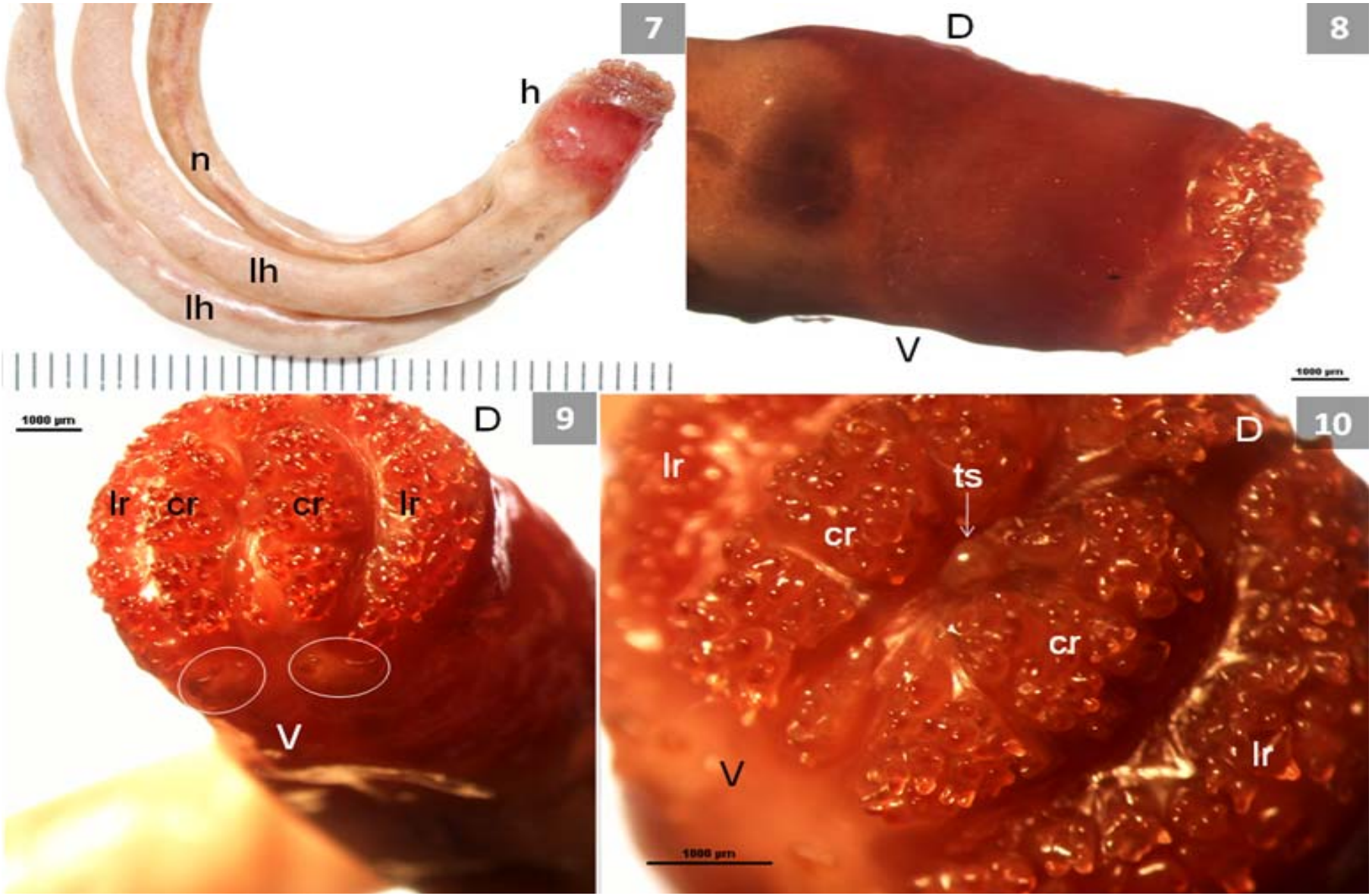
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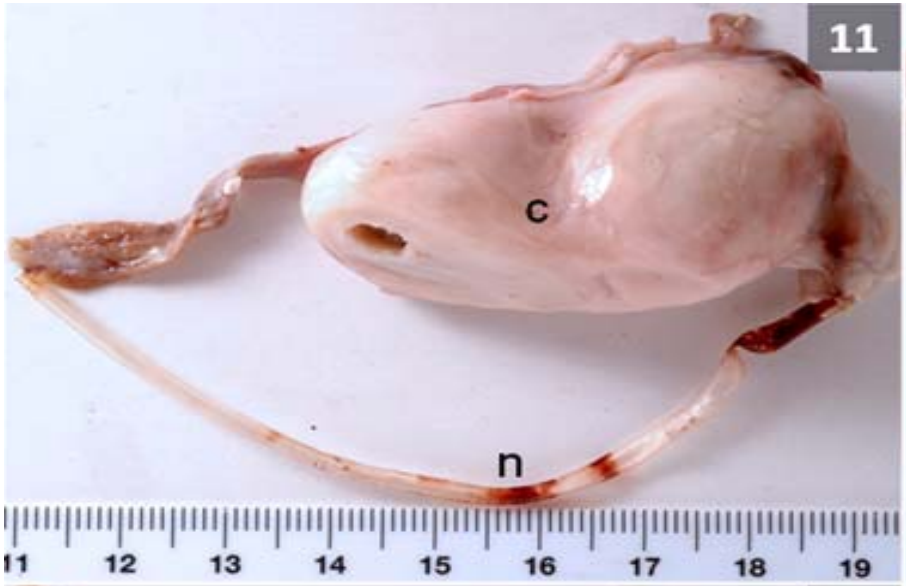
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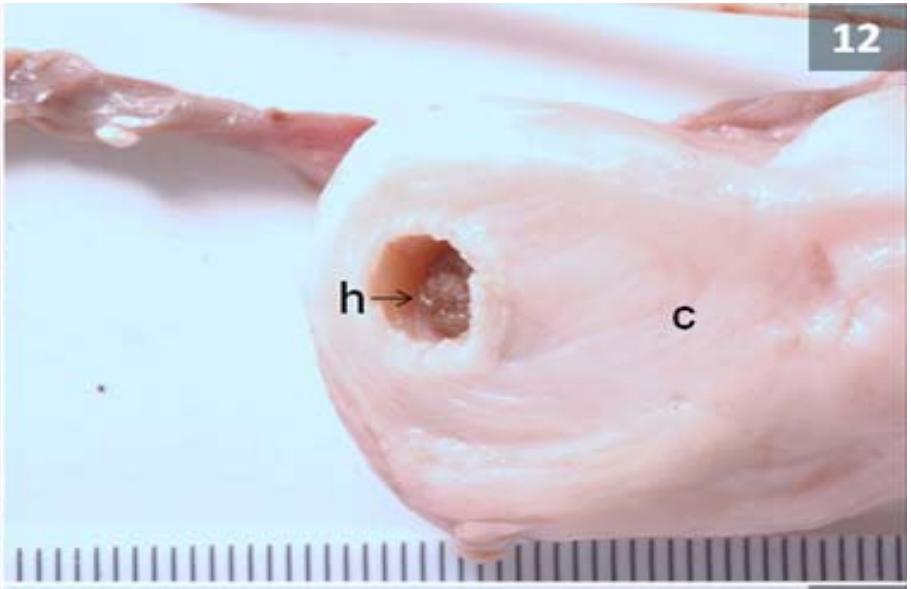
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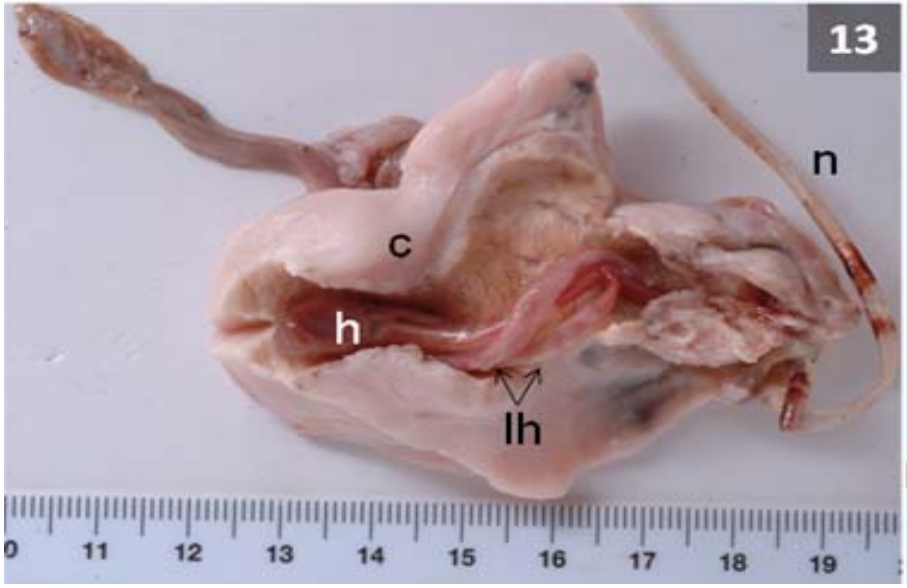
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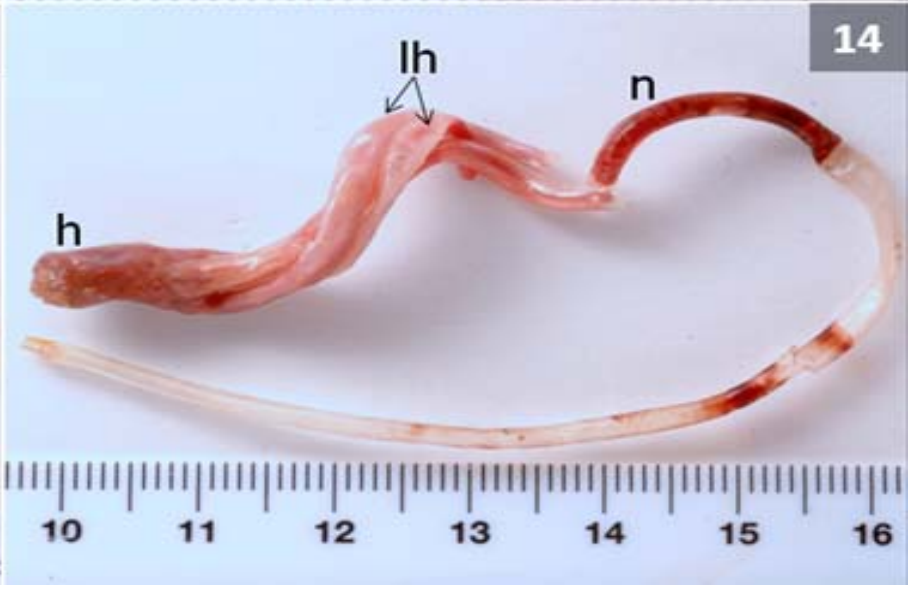
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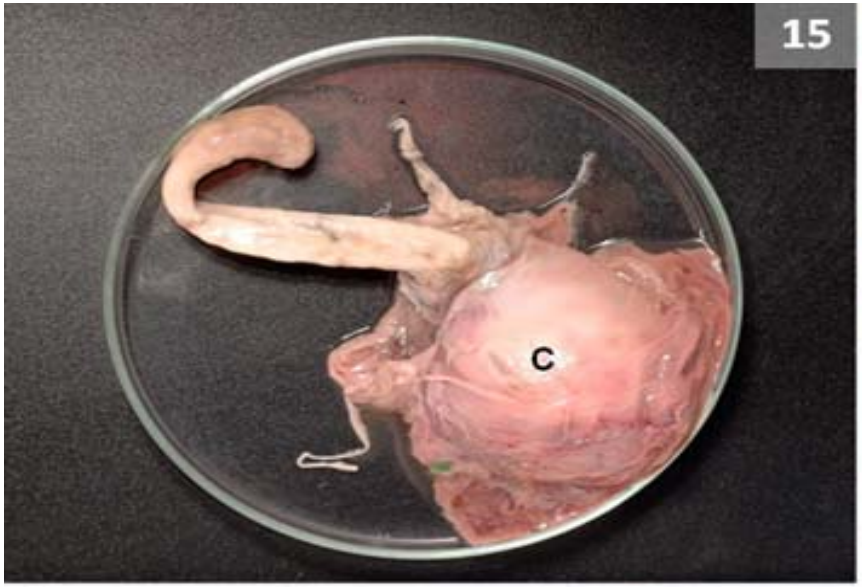
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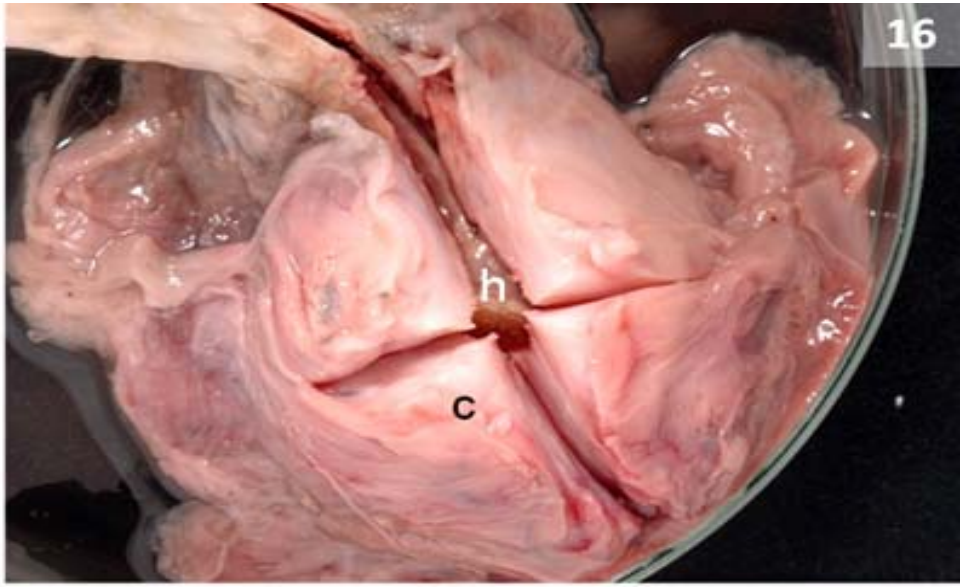
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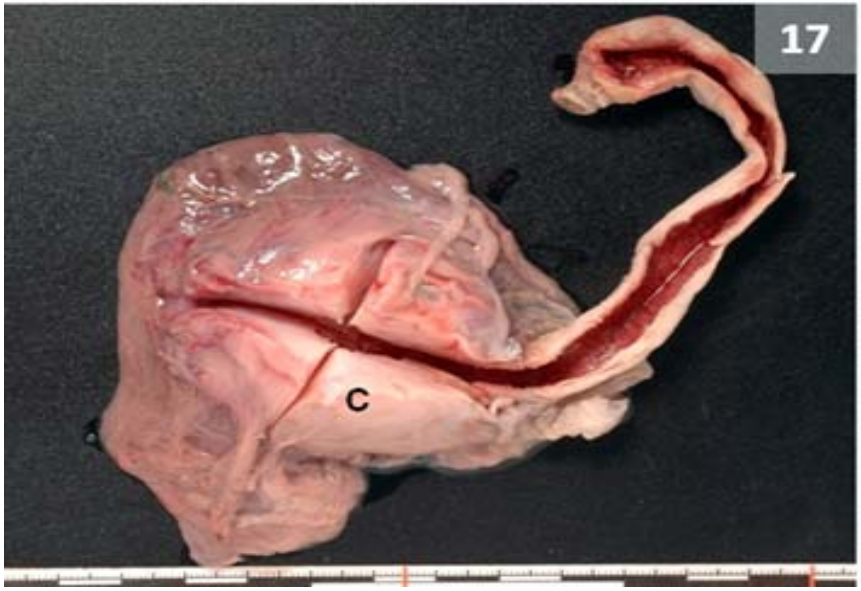
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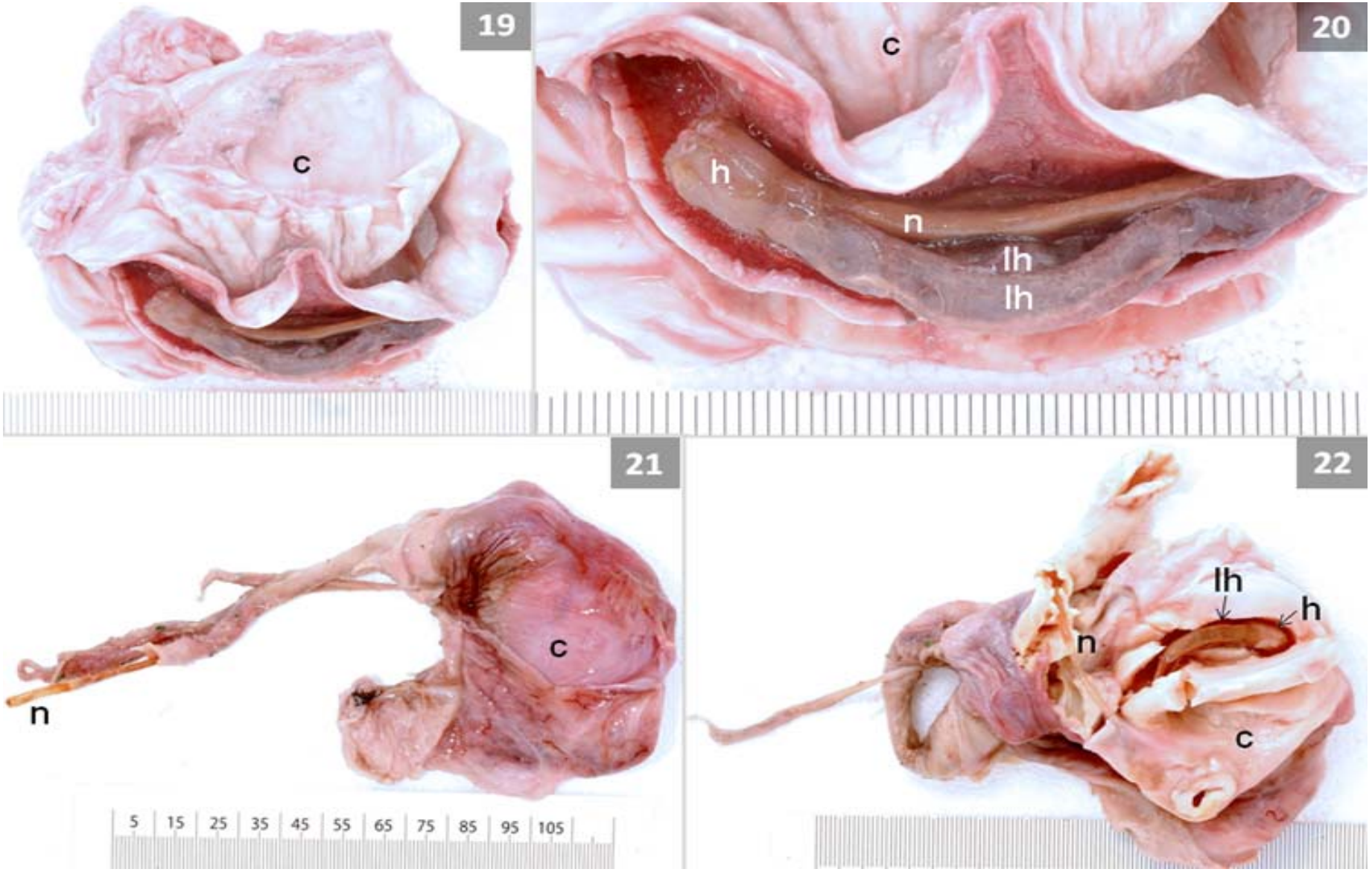
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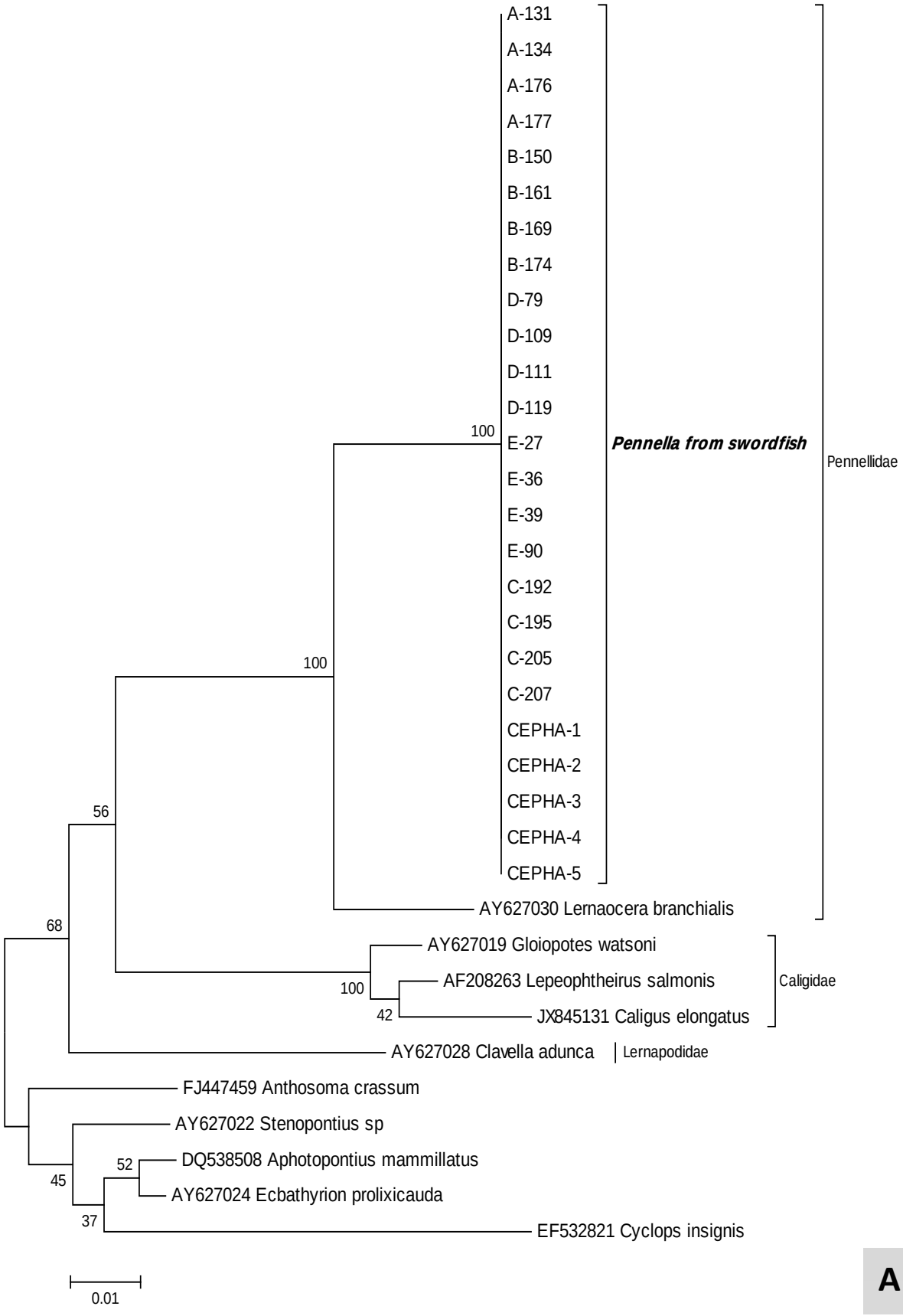
Figure 8.5. (1-22). General and macro photographs of five *P. instructa* cephalothoraxes. **(1-10)** CEPHALOTHORAX 1. **1**: Cyst (c) and posterior external portion which includes neck (n), trunk (t), abdominal brush (ab) and egg strings (arrows). **2**: Detail of trunk (t), abdominal brush (ab), egg strings (arrows) and plumes (p). **3**: Detail of the head (h) inside the dissected cyst. **4-5**: Dissected cyst (c) with parasite. **6**: Dissected cyst (c) and anterior end of extracted parasite. Head (h), fragmented neck (n) and lateral horns (lh). **7**: Detail of anterior end with head (h), neck (n) and lateral horns (lh). **8-10**: Stereomicroscope views of the head. Ventral and dorsal faces (V, D), single papillae (white circles), central and lateral rows of papillae (cr, lr), and trilobed structure (ts) observed on head's anterior surface. **(11-14)** CEPHALOTHORAX 2. **11**: Neck (n) emerging at the right end of cyst (c). **12**: Detail of the head (h) within the cyst. **13**: Dissected cyst (c) with parasite. Head (h), neck (n) and lateral horns (lh). **14**: Anterior end of parasite. Head (h), neck (n) and lateral horns (lh). **(15-18)** CEPHALOTHORAX 3. **15**: Cyst (c). **16**: Dissected cyst (c) with parasite. Head (h). **17**: Dissected cyst (c) without parasite. **18**: Anterior end of parasite. Head (h), segmented neck (n) and lateral horns (lh). **(19-20)** CEPHALOTHORAX 4. **19**: Dissected cyst (c) with parasite. **20**: Detail of anterior end inside the cyst (c). Head (h), neck (n) and lateral horns (lh). **(21-22)** CEPHALOTHORAX 5. **21**: Neck (n) emerging at the left end of cyst (c). **22**: Dissected cyst (c) with parasite. Head (h), neck (n) and lateral horn (lh).

8.3.3. Molecular identification

PCR amplification and DNA sequencing for 18S and 28S rRNA gene regions was successful. The sequences obtained for each gene showed 100% identity among them. BLAST searches of 18S sequences (785 bp) showed moderate identities to *Lernaeocera branchialis* (95%) and *Anthosoma crassum* (94%). BLAST searches of 28S sequences (326 bp) showed a lower identity of 87% to *Paracyclopina nana* and 86% to *Caligus curtus*.

The taxonomic affiliation of the *Pennella* parasite infecting swordfish was determined by phylogenetic analyses of 18S and 28S rRNA gene. The tree constructed using ML method for 18S sequences showed that all sequences of *Pennella* parasites from swordfish were placed together (bootstrap values 100%) and in the same clade with *Lernaeocera branchialis* with high bootstrap value (100%) (Figure 8.6.A). Members of the families Pennellidae, Caligidae and Lernapodidae clustered together with bootstrap values of 68%. For 28S sequences, ML method placed unequivocally all sequences of *Pennella* from swordfish together with bootstrap values of 100%. The tree showed that *Pennella* clustered with members of the *Cyclopoida* and *Harpacticoida* and showed a most distant relationship with other members of the *Siphonostomatoida*, which are grouped in other clade with bootstrap values of 88%.

These results revealed that only one pennellid species, *Pennella instructa*, was identified by molecular studies as responsible for swordfish infection in the total fishes examined, including external fragments, and cephalothoraxes enclosed in parasitic cysts from the five geographic Atlantic areas defined (Table 8.1).


A-131
A-134
A-176
A-177
B-150
B-161
B-169
B-174
D-79
D-109
D-111
D-119
E-27
E-36
E-39
E-90
C-192
C-195
C-205
C-207
CEPHA-1
CEPHA-2
CEPHA-3
CEPHA-4
CEPHA-5
Pennella from swordfish
Pennellidae
AY627030 Lernaocera branchialis
AY627019 Gloiopotes watsoni
AF208263 Lepeophtheirus salmonis
JX845131 Caligus elongatus
Caligidae
AY627028 Clavella adunca
Lernapodidae
FJ447459 Anthosoma crassum
AY627022 Stenopontius sp
DQ538508 Aphotopontius mammillatus
AY627024 Ecbathyrion prolixicauda
EF532821 Cyclops insignis
100
100
56
68
100
42
52
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37
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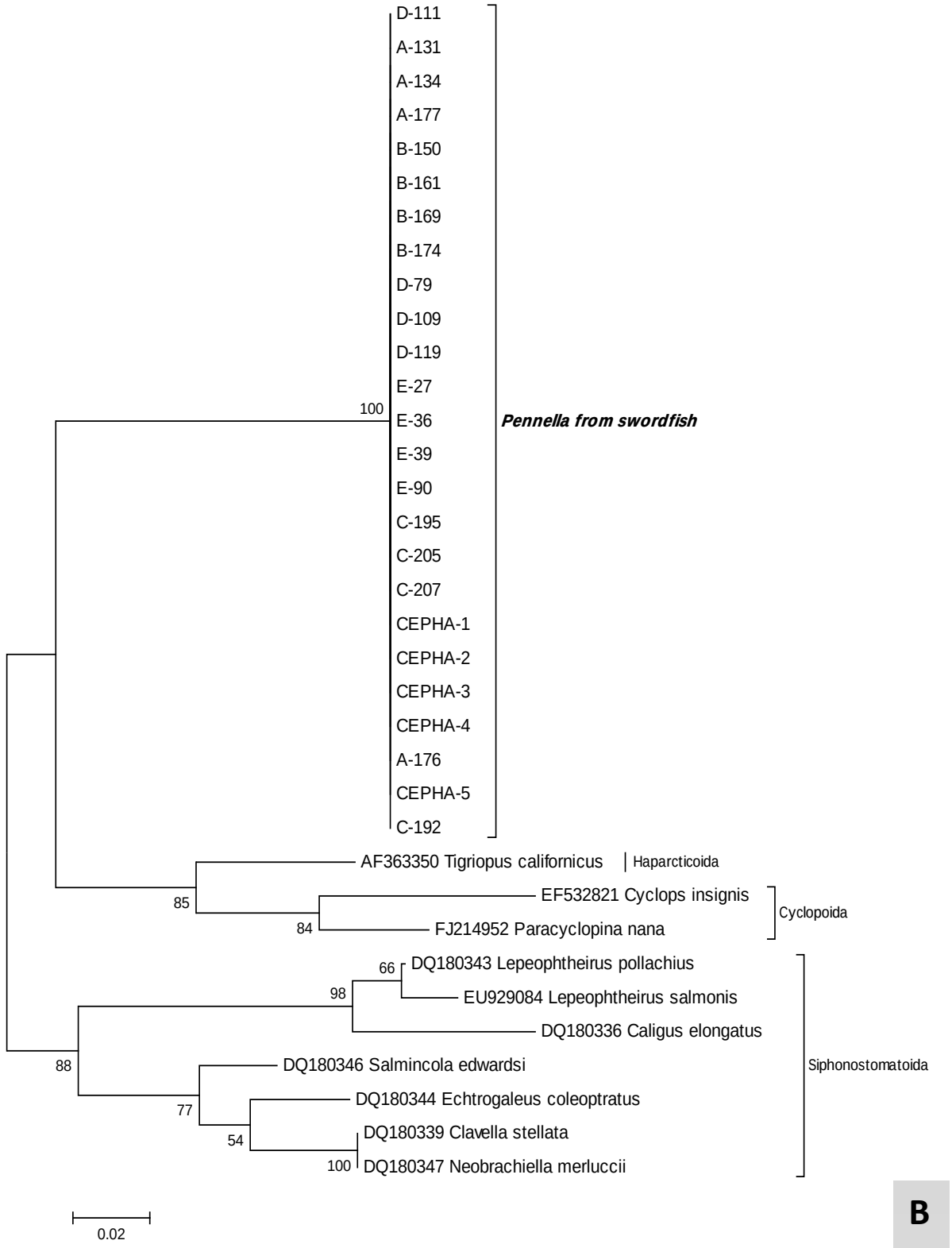

Figure 8.6. (A-B). Phylogenetic analysis inferred from Maximum likehood analysis of partial 18S **(A)** and 28S rDNA **(B)** sequences showing the taxonomic position of *Pennella* parasite infecting *Xiphias gladius* in relation to other copepods. Numbers at branch nodes indicate bootstrap confidence values in percent. Pennellid samples analysed are coded as "fishing area (except for cephalothorax) - parasite code", as stated in Table 8.1.

8.3.4. Demography of infection

A value of Prevalence of infection P=10.24±0.74% for *P. instructa* was determined in the swordfishes examined in the fish auction market, since from the total individuals checked, 167 were identified as infected hosts. In addition, the maximum number of *P. instructa* specimens on a swordfish was 4, thus mean Intensity (I) value of infection obtained was 1.28±0.7 parasites per infected host.

Moreover, pennellids were widely distributed on hosts' body. For a better understanding and in order to illustrate more clearly which parts of the swordfish were affected and their level of intensity, the number and percentages of the external pennellid portions collected, according to their anatomical site of infection, have been situated in a swordfish body scheme (Figure 8.7).

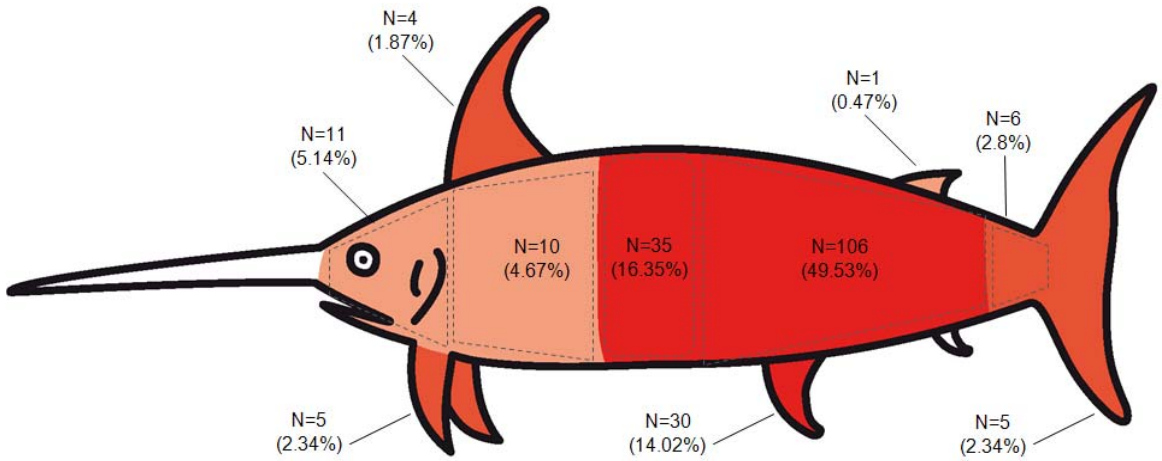

Figure 8.7. Swordfish's body scheme of the distribution by anatomical regions of the total external pennellids collected. Intensities of red colour indicate higher or lower quantities of parasites. Total number and percentages per anatomical region have been appropriately situated.

8.4. DISCUSSION

In accordance with the detailed redescription of *P. instructa* published in 1986 by Hogans, and on the basis of the morphological information extracted from the present work, most anatomical characters of pennellids concerning neck, trunk, abdomen or plumes, which could not be measured due to the impossibility of obtaining complete parasites, did not differ significantly from other specimens previously described, neither from the original description (Wilson, 1917). However, as Hogans reported in the cited publication, the age of the parasite and the site of attachment in the host are probably responsible for the variation in some body features among *Pennella* specimens. Specifically, with regard to head characters,

noticeable disparities were found between species. *P. charcoti*, was originally described by Quidor (1913) presenting a clove-shaped head. Years later, Abaunza et al. (2001) reported a "globose" head on *P. balaenoptera.* A wide study on the same species carried out by Hogans (1987) described the head as a sub-spherical to sub-cylindrical structure. In addition, this last author added the ovoid shape to the cephalothorax of *P. fillosa*. However, similarly to one of the two specimens morphologically redescribed by Hogans in 1986, cylindrical conformation was observed in all the specimens of *P. instructa* studied in the present work. Moreover, in line with the description that this author carried out in the cited paper on *P. instructa*, two single papillae have been found here, below the head on the ventral face. Also in relation to the head, in contrast with the anatomical aligned distribution of the antennary processes previously described by Hogans (1986), which is also present in our cephalothoraxes, other descriptions of *P. fillosa* and *P. balaenoptera* (Hogans, 1987; Benz and Hogans, 1993) reported a wide variability in shape, size and configuration of this antennary processes. Specifically, Abaunza et al. (2001) observed the presence of two big groups of papillae covering the anterior end of cephalothoraxes of *P. balaenoptera.*

Moreover, if comparing different species of *Pennella,* many variations can be observed in the anatomical conformation and number of the cephalothoric lateral horns. In the case of *P. balaenoptera* the two lateral horns are often extremely long (Hogans, 1987; Abaunza et al., 2001). However, in *P. fillosa* these two structures have been observed short and emerging immediately posterior to bulbous portion of cephalothoraxes, perpendicular to axis and opposite one another (Hogans et al., 1985; Benz and Hogans, 1993). Additionally, the presence of a smaller bifurcate dorsal horn at level of lateral horns was firstly described in *P. crassicornis* by Wilson (1917), and also reported for *P. fillosa* by Benz and Hogans (1993) and *P. balaenoptera* (Hogans, 1987). Nevertheless, Abaunza et al. (2001) suggested that dorsal horn is not present in all the cases. Although some of these authors stated that the length, presence/absence of holdfast horns are directly related to site of attachment in host, in the cases referred here the presence of the same number, an identical position in the head and proportional lengths of lateral horns among specimens, were noticed.

During slices inspection, any pennellid was seen situated with the cephalic portion attached near a blood vessel or burrowed deeply into areas with a rich blood supply. Cephalothoraxes were not even located in the surrounding areas of visceral cavity. This finding contrasts with the statement published by Mattiucci et al. in 2005.

Despite the documented plasticity of Pennellidae (Hogans, 1987; Benz and Hogans, 1993; Brooker et al., 2007; Abaunza et al., 2001), in accordance with the morphological characters previously described by Hogans (1986) and herein observed, and on the basis of genetic results, it may be suggested that populations of *X. gladius* in the sampling areas (including both cephalothoraxes and parasites from swordfish sampled in the fish auction market), were infected by a pennellid species, *P. instructa*. The

"parasite-host" anchorage scheme observed suggested that slices were also parasitized by this species, *P. instructa*.

Impact on fish

Although the pathology of pennellid infections has to be more deeply studied (Hogans et al., 1985), in the present work it has become evident that these parasites are often (23.84% of cases) found anchored in the fins and fin insertion areas of swordfish, thus damaging the swimming muscles or compromising their ability to swim. Moreover, their presence emerging along the body, predominantly on the posterior part, even occupying natural orifices, becomes a problem for hosts since their development and well-being are compromised. Although the present work has not been able to demonstrate it, other authors have stated that these mesoparasites could also threat vital functions by harming internal organs such as the heart, aorta or other blood vessels, ovary, intestine or stomach (Hogans et al., 1985; Hogans, 1986; Brooker et al., 2007; Damiano, 2007). Furthermore, the pressure-induced damage from the high size of cysts that usually are formed in the musculature, often near the vertebral column, as consequence to the presence of these mesoparasites, may also affect the hosts' nervous system.

The commercial impact of *Pennella* spp. in fish quality has prompted serious concern within the fisheries sector, and consequently among scientists and public health inspectors, mostly due to the applicability of Regulation EC 178/2002, which establishes that for reasons of contamination fish infected with visible parasites is unfit for human consumption. The presence of pennellids as the most important macro parasite affecting *X. gladius*, usually involves the formation of evident cysts and the deterioration of organoleptic properties as direct consequence (Lester et al., 1995; Damiano, 2007). In this regard it would be helpful to have studies about costs of these parasitism in the short and medium-term, in order to improve our understanding of the problem and to undertake preventive and corrective measures. Moreover, monitoring actions and proactive self-inspections should be integrated into daily work with the purpose of preventing damage on physical characteristics of fishes, and ensuring safer and high quality standard products to final consumers.

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CHAPTER 9

Inspection Scheme

SADE: A parasite scoring system for fish assessment

Llarena-Reino, M., Abollo, E. and Pascual, S. (2013). A scoring system approach for the parasite predictive assessment of fish lots: a proof of concept with anisakids. Foodborne Pathogens and Disease, 10(12):1067-1074.

ABSTRACT

A total of 982 individuals distributed in 11 lots belonging to 10 fish species from three Atlantic FAO fishing areas were sampled and examined to detect the presence of anisakid larvae in fish muscle. After hazard identification by genetic sequencing and exposure assessment by anatomic extent and demographic characterization of infection, all data were fitted for each fish species to a new proposed scoring schema of parasite prediction. In the absence of a criterion standard method for inspection and precise definition of the "*quantum satis"* for parasites in contaminated fish lots, the inspection-rating scheme called SADE (Site of infection, Assurance of quality, Demography, Epidemiology) may help fish industries to precisely handle and to evaluate the likely outcome of infected fish lots after being diagnosed. For this purpose, a supporting flow diagram for decision was defined and suggested. This new performance assessment tool has the aim of staging fish lots, thus helping in planning manufacture, commercial, and research decisions during self-management programs. This novel scoring system provides an improved inspection format by implementing the occurrence stratification for parasites to guide Hazard Analysis and Critical Control Points (HACCP) programs for the uniform exchange of information among fish industries, administration and researchers, thus facilitating standardization and communication. In the future, this scoring version could be validated (in terms of classification and wording) for similar overall predictive purposes in other muscular parasites infecting seafood products.

KEYWORDS

Scoring system; parasite; fish; SADE; HACCP

9.1. INTRODUCTION

Since the mid-20th century, scientific evidence has confirmed the presence of L3 anisakid larvae in a high and rising number of fish species of commercial interest around the world (Smith and Wootten, 1979; McClelland et al., 1985; Adams et al., 1997; Abollo et al., 2001; Rello et al., 2009). The presence of this parasite causes clinical infections and sometimes produces panzootic fish diseases. Anisakid parasites represent the target tip of a ''dirty list'' of parasites found in seafood during veterinary inspections, with increasing records in the Rapid Alert System for Food and Feed System. The economic losses and public health concern caused by the visual impact of both alive and dead anisakid worms decrease the commercial value of fishes (Vidacek et al., 2009). The recognized effects on human health of these emergent zoonoses (causing symptoms ranging from gastrointestinal disorders and allergic diseases in consumers to occupational asthma in fish-farming workers) (Smith and Wootten, 1978; Dick et al., 1991; Audicana et al.,

2002; Plessis et al., 2004; Nieuwenhuizen et al., 2006; Chen et al., 2008; Vidacek et al., 2009) were recently recognized by the Panel on Biological Hazards of the European Food Safety Authority (EFSA, 2010).

Most anisakid larvae are found in the viscera, mesentery, and gonads of the fish (Vidacek et al., 2009), and in a lower amount in the flesh (Wharton et al., 1999; Llarena-Reino et al., 2012). The number of muscular anisakids depends basically on the ecological niche of fish species (Holst et al., 1993; Stromnes and Andersen, 1998). It has been noted that there is some *post-mortem* migration of the larvae from the viscera cavity into flesh (Smith, 1984), although it is not clear when, under what conditions, and in which fish species this occurs (EFSA, 2010).

Invasive fish inspection methods are currently considered ‘‘better’’ or ‘‘truer’’ because they allow direct examination of flesh parasites and their spread in the edible part of fish, in contrast to nondestructive methods, which in the case of whole fish are clearly limited by the fact that the information is obtained by making indirect observations at parasites in the gut (Commission Regulation (EC) 2074/2005), resulting in biased estimations with no statistical confidence (Llarena-Reino et al., 2012). Several methods have been developed and used for detection, diagnosis, and identification of parasites in fish, from the oldest ones such as visual inspection (Hartmann and Klaus, 1988), light microscopy (Rijpstra et al., 1988), or candling (Wold et al., 2001; Butt et al., 2004), until some revised and recently updated ones such as the pepsin digestion protocol (Lysne et al., 1995; Lunestad, 2003; Thien et al., 2007; Thu et al., 2007; Llarena-Reino et al., 2013). These methods are being applied by fishery operators or laboratories. Recent techniques including ultraviolet illumination (Adams et al., 1999; Levsen et al., 2005; Marty, 2008), ultrasound (Hafsteinsson et al., 1989; Nilsen et al., 2008), X-rays and conductivity (Nilsen et al., 2008), electromagnetism (Choudhury and Bublitz, 1994), magnetometry (Jenks et al., 1996), immunological techniques (Xu et al., 2010; Rodríguez-Mahillo et al., 2010), polymerase chain reaction (PCR)-based (Zhu et al., 2002; Abe et al., 2005; Pontes et al., 2005), real-time PCR (Herrero et al., 2010; Fang et al., 2011), phage display (López et al., 2011), real-time fluorescence resonance energy transfer (Monis et al., 2005; Intapan et al., 2008), or imaging spectroscopy (Heia et al., 2007) are under continuous improvement processes.

Regardless of the inspection method employed, when facing up to an infected fish lot, corrective measures settled at any step or procedure will depend on how relevant the parasite infection is. In other words, the Hazard Analysis and Critical Control Points (HACCP) works in an overall predictive assessment fashion that should include the parasite identity, the spread of parasites in the edible part of fish, and the food quality and safety implications of this biological hazard. This study was intended to help express and resolve all these questions by designing a simple scoring system of parasite infection in fish flesh. In order to provide evidencebased criteria, we inspected and then scored several commercial frozen fish lots to offer a proof-of-concept of the applicability of the inspection system proposed.

Table 9.1. Data from the fish lots studied including their FAO origin areas, ranges of length and weight, and demographic values of anisakid infection (P, I, A, and D)[a]

FAO Fishing Areas	Fish species	Individuals (N)	Total Length Range (cm)	Total Weight Range (g)	Prevalence (% ± CI)			Mean Intensity (± SD)			Mean Abundance (± SD)			Density			SADE Code	Score
					Epaxial	Hypaxial	Total	Epaxial	Hypaxial	Total	Epaxial	Hypaxial	Total	Epaxial	Hypaxial	Total		
21 (NAFO), Div 3M	*Macrourus berglax*	50	37-60	272-1586	0	34±6.56	**34±6.56**	0	3.88±5.19	**3.88±5.19**	0	1.32±3.56	**1.32±3.56**	0	5.25	**5.25**	S2 A1 D0 E0	**3**
41 (3.2. South Patagonia), Malvinas	*Macruronus magellanicus*	17	44-82	255-1573	0	35.29±11.35	**35.3±11.35**	0	1.83±1.2	**1.83±1.2**	0	0.65±1.06	**0.65±1.06**	0	1.15	**1.15**	S2 A2 D2 E0	**6**
27 (NEAFC), Div VIb	*Micromesistius poutassou*	50	25.5-38	84-296	6±3.29	78±5.74	**78±5.74**	1	4.02±3.99	**4.1±3.98**	0.06±0.2	3.14±3.91	**3.2±3.91**	0.55	28.75	**29.3**	S0 A1 D0 E0	**1**
27 (ICES), Div XII	*Coryphaenoides rupestris*	50	44-95	229-1956	0	6±3.29	**6±3.29**	0	1.3±0.58	**1.3±0.58**	0	0.08±0.34	**0.08±0.34**	0	0.224	**0.224**	S2 A2 D2 E0	**6**
27 (ICES), Div XIVb	*Sebastes mentella*	50	29.8-44	287-856	0	11.8±4.47	**11.8±4.47**	0	6.66±5.8	**6.66±5.8**	0	0.78±2.96	**0.78±2.96**	0	2.4	**2.4**	S2 A1 D1 E0	**4**
27 (ICES), Div VIIIc	*Micromesistius poutassou*	329	21.5-28.5	52-172	8.17±1.48	54.21±2.69	**55.73±2.7**	1.88±1.79	4.39±11.28	**4.56±11.43**	0.16±0.67	2.94±8.68	**3.1±8.91**	2.55	47.54	**50.1**	S0 A1 D0 E0	**1**
27 (ICES), Div VIIIc	*Scomber scombrus*	236	27-43	123-645	3.45±1.16	26.48±2.81	**28.92±2.9**	1	2.2	**2.15±2.4**	0.03	0.58	**0.62±1.5**	0.14	3	**3.15**	S0 A2 D1 E0	**3**
27 (ICES), Div VIIh	*Lepidorhombus whiffiagonis*	50	21.5-26.5	74-153	10±4.15	20±5.54	**28±6.22**	1±0.5	1.7±0.95	**1.57±0.85**	0.1±0.36	0.34±0.8	**0.44±0.84**	1.41	4.8	**6.2**	S0 A2 D0 E0	**2**
27 (ICES), Div VIIj	*Lophius budegassa*	50	35.5-52.5	571-1909	6.12 ± 3.3	91.83±3.79	**93.88±3.3**	1	16.15±35.49	**15.87±35.62**	0.06±0.24	14.84±34.25	**14.9±34.38**	0.13	30.56	**30.69**	S0 A1 D0 E0	**1**
27 (ICES), VII h	*Lophius piscatorius*	50	26-38	269-826	10±4.15	64±6.65	**68±6.46**	1.6	2.66±2.67	**2.73±2.66**	0.16±0.14	1.7±2.57	**1.86±2.57**	1.1	11.69	**12.79**	S0 A1 D0 E0	**1**
27 (ICES), Div VIIj	*Merluccius merluccius*	50	34-53	215-785	14±4.8	90±4.15	**90±4.15**	3.14	85.6±192.67	**86.13±192.7**	0.44±0.2	77.08±184.4	**77.52±184.5**	1.45	252.9	**254.4**	S0 A1 D0 E0	**1**

[a] The resulting SADE code and the final score for each lot after applying the staging system proposed here are also provided. P, I, A, and D: Prevalence, mean Intensity, mean Abundance, and Density. CI: confidence interval. SD: standard deviation. SADE (scoring system): Site of infection, Assurance of quality, Demography, Epidemiology.

9.2. MATERIALS AND METHODS

9.2.1. Parasite diagnosis

As Regulation (EC) 2074/2005 specifies in Section 1 of Annex II, laying down specific provisions for visual inspection of eviscerated fish, fish fillets and slices, a representative number of individuals will be submitted to a visual inspection at establishments on land and on board factory vessels. It also states that qualified technicians from establishments will determine the scale and frequency of inspections depending on the type of the fish products, their geographical origin, and the final use they are intended for. During the present work and as a proof-of-concept to demonstrate the feasibility of this scheme to be incorporated to routine quality control programs in fish industries, a total of 11 commercial lots belonging to 10 fish species, each one comprising 17-329 specimens from three FAO fishing areas, were sampled and characterized as summarized in Table 9.1. The whole musculature of each individual was inspected. Guts were not included in the examinations because these parts are usually discarded during fish-processing procedures. At the time of capture, fishes were frozen at -20°C in order to avoid migrations of anisakid larvae from visceral cavity to somatic muscle. Full necropsies, collection of parasites, and tissue sampling were carried out in every single fish. Then, each fish was thinly sectioned and every fragment was visually inspected for parasites on a candling table with the aid of a Nikon SMZ800 stereomicroscope. Afterward, the whole fish muscle (hypaxial and epaxial regions separately) of each individual was digested in pepsin solution (according to Llarena-Reino et al., 2013) to recover previously undetected parasites during the visual inspection. Any parasite found was identified on the basis of morphoanatomical diagnostic characters (Berland, 1961 and 1989; Fagerholm, 1982; Olson et al., 1983; Smith, 1983; Køie, 1993). Moreover, for some specimens molecular identification was performed by amplification and sequencing of the ITS1-rDNA region, using the primers NC5-NC2 (Zhu et al., 1998). DNA extraction of nematodes was carried out with NucleoSpin Tissue Kit (Macherey-Nagel). PCR reactions were performed in a total volume of 25 µL containing 1 µL of genomic DNA (150–200 ng), PCR buffer at 1 x concentration, 1.5mM $MgCl_2$, 0.2mM nucleotides (Roche Applied Science), 0.3 µM primers, and 0.625U Taq DNA polymerase (Roche Applied Science). The cycling program was 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, and 75 s at 72°C, followed by 7 min at 72°C. PCR products were separated on a 1% agarose (in 1 x Trisacetic EDTA buffer) gel, stained with ethidium bromide, and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories). PCR products were purified with MinElute PCR Purification Kit (Qiagen GmbH, Hiden, Germany). Sequencing was performed by Secugen Company (Madrid, Spain). The chromatograms were analyzed using ChromasPro v.1.41 (TechnelysiumPty Ltd.). Sequences were subject to Basic Local Alignment Search Tool (BLAST) analyses against available sequences from GenBank, through web servers of the National Center for Biotechnology Information (USA). The terms prevalence (P), mean intensity (I), mean abundance (A), and density (D) of infection were determined for each fish lot following Bush et al. (1997) and Rózsa et al. (2000).

9.2.2. Scoring system

The scoring system, namely SADE (Site of infection, Assurance of quality, Demography, Epidemiology), proposes the categorization of fishes/lots infected by parasites. This tool is being presented in a highly visual and rapid-reference format. Fish lots are grouped according to four homogeneous categories (indices or ''bins'' of disease importance, namely S, A, D, and E), which are further divided with some accommodation into subcategories (denoted by numerals). The lower the number, the more advanced the hazard (i.e., ''high-risk features'') tends to be. The objective of SADE is the score of fish lots. By summing the numerical values assigned to each batch along the four categories, the SADE system adopts a 10-point scale. Each company must determine the level of score that sets off the implementation of measures to ensure food safety and quality of processed batches. The highest score indicates parasite-free fish lots. The lowest scores refer to serious weaknesses in the fish evaluated; that means a fish lot that should be reprocessed to guarantee its visual quality and/or safety attributes.

- ***S****ite of infection (the S category assesses the anatomic exposure of fish flesh recorded at inspection).*

  S0: disseminated (spread throughout the whole flesh)

  S1: located in the epaxial zone

  S2: located in the hypaxial zone, including the visceral body cavity

  S3: parasite-free

- ***A****ssurance of quality: macroscopic pathological-unaesthetic commercial findings (the A category shows whether there are manufacturing and/or visual parasite problems reported at line or on site in contaminated fish lots).*

  A0: both topics included in A1 (pathological changes and parasite motility)

  A1: gross pathological changes in infected tissues (undesirable components such as nodules in bellyflaps, melanized capsules in fillets, milky flesh, hemorrhages in the vent areas (e.g., Beck et al., 2008) or commercial reject due to a live parasite, mostly associated with parasite motility in fresh fish (e.g., Pascual et al., 2010)

  A2: neither pathological nor commercial problems

- ***D****emography of infection (the D category assesses the quantity of infection recorded at inspection, upon adapted and combined criteria based on CODEX STAN 165 [1989], CODEX STAN 190 [1995], CX/FFP 08/29/7, and on Wooten and Cann [2001]).*

D0: density > 5 parasites/kg

D1: density 2–5 parasites/kg

D2: density < 2 parasites/kg

- ***E**pidemiological relevance of the species (the E category describes the risk of the hazard after parasite species diagnoses, based on EFSA opinion and previous clinical evidences, already cited).*

  E0: zoonotic species of parasite (or its metabolites) associated with gastrointestinal diseases, other documented allergies, and/or clinical manifestations

  E3: species of parasite with no published evidence-based data demonstrating human health affection. The importance of this point in terms of food security leads to assigning it a value of 3 points.

9.2.3. Flow diagram: An easy tool to use the scoring system

Based on the SADE scoring system and following an HACCP schema, the flow diagram herein proposed was subsequently generated to standardize epidemiological stages provided by fish-inspection results. Figure 9.1 illustrates this flow diagram as a user-friendly tool that can be easily implemented and controlled by the technicians and followed by fish workers.

9.3. RESULTS

9.3.1. Parasite diagnosis

Table 9.1 gathers the characteristics of all the processed fish lots. Three nematode species belonging to *Anisakis* and *Pseudoterranova* genera were identified by molecular studies as responsible for muscular infection in the fish lots analyzed (Table 9.2). For every fish species, demography of infection showed higher values at the hypaxial region than in the epaxial muscle (Figure 9.2). In fact, over 45% of inspected lots were parasite free at the epaxial muscle, whereas all the lots showed some degree of infection at the belly-flap region surrounding the viscera (hypaxial region). Anisakid parasites were never exclusively found in epaxial flesh. Although these results showed that epaxial infection always took place simultaneously with hypaxial location and not vice versa (this may be related to migration routes from viscera to muscle), some authors have demonstrated that there is a positive relationship between the gut and muscular number of parasites at epaxial musculature as well (Llarena-Reino et al., 2012). Because of this, epaxial infection has to be taken into account during fish inspection processes. On the other hand, demographic values of parasite infection were the highest (from high to low) in *Lophius budegassa, Merluccius merluccius, Micromesistius poutassou*,

and *Lophius piscatorius. Coryphaenoides rupestris* showed the lowest anisakid infection values. No fish species were found to be free of parasites.

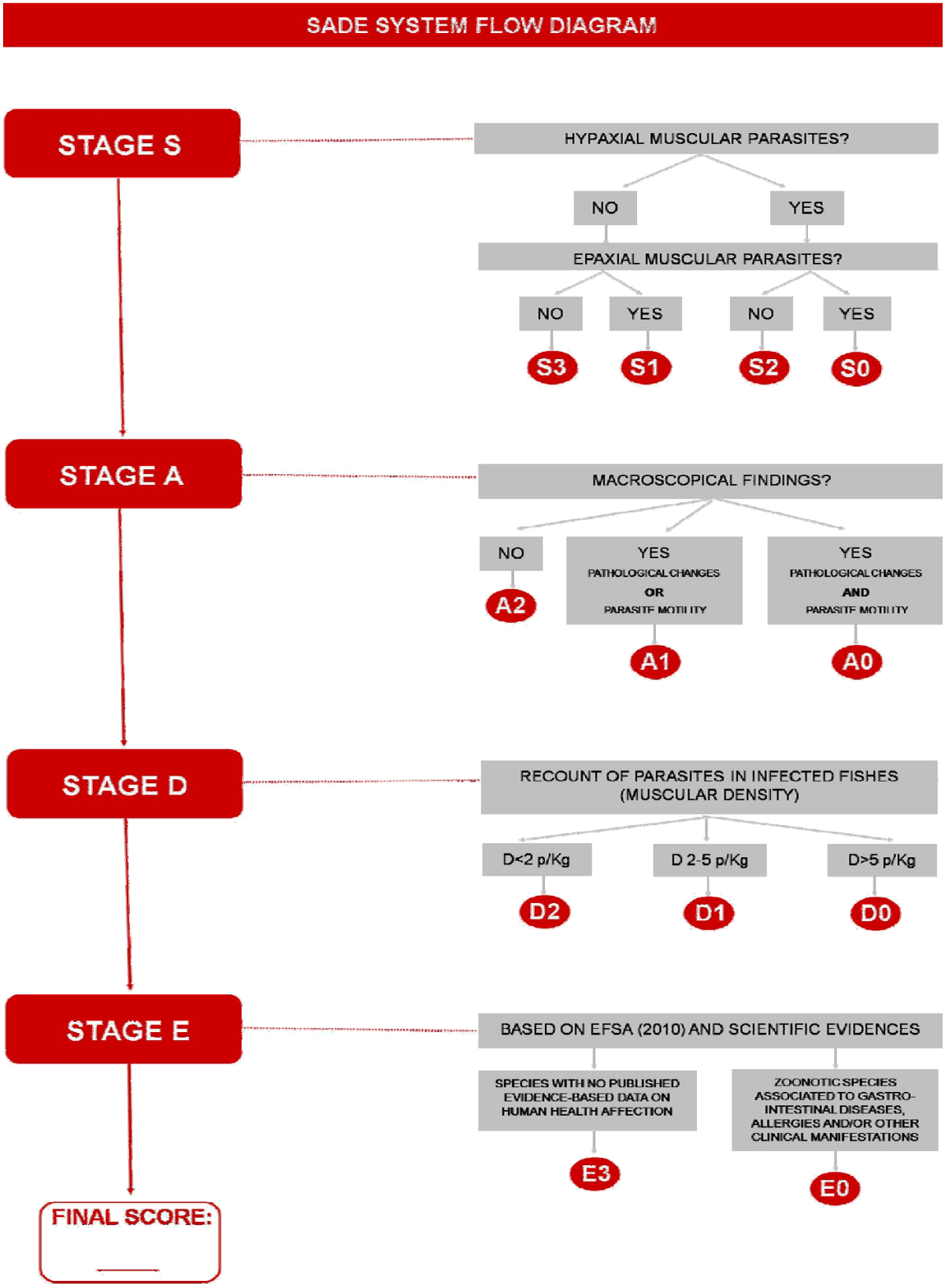

Figure 9.1. Flow diagram for the Site of infection-Assurance of quality-Demography-Epidemiology (SADE) Scoring System illustrates an ordered and structured work schema based on Hazard Analysis and Critical Control Point principles to be easily implemented and followed by fish industries. From stage 1 to 4, the user classifies each inspected fish lot according to the localization of parasites, the presence/absence of pathological or unaesthetic signs in the edible part of fishes, the density of infection, and finally to the epidemiological relevance of the etiologic agent. As result, a SADE code and a final score are obtained for each lot checked, in order to decide which industrial process or final destination may be followed.

9.3.2. Fitting the scoring system

Results based on epidemiological relevance of the parasite, pathological findings, and demographic values of infection for each fish lot fit easily into the scoring strategy. Table 9.1 reports the inspection results categorized by the SADE scoring system, thus showing for each fish species a ‘‘SADE Score’’ as results of the addition of the code points. For example, *Merluccius merluccius* from FAO 27 has a scoring of 1, which results after adding up the scoring in each code (‘‘S0 A1 D0 E0’’). The score refers to a fish lot with a disseminated *Anisakis* infection, which could produce gastrointestinal diseases, allergies, and/or other clinical manifestations to consumers, relevant commercial repercussions (due to evident pathological signs in the infected areas), and density values of infection greater than five parasites per kilogram. Regarding the resulting scores, all species had between 1 and 6 points, and FAO 27 species (except for *Coryphaenoides rupestris* and *Sebastes mentella*) were the lowest scoring species. It also was remarkable that the two lots whose fishes showed the largest body lengths (belonging to *Coryphaenoides rupestris* and *Macruronus magellanicus*, from higher to lower) were the groups with the highest resulting scores.

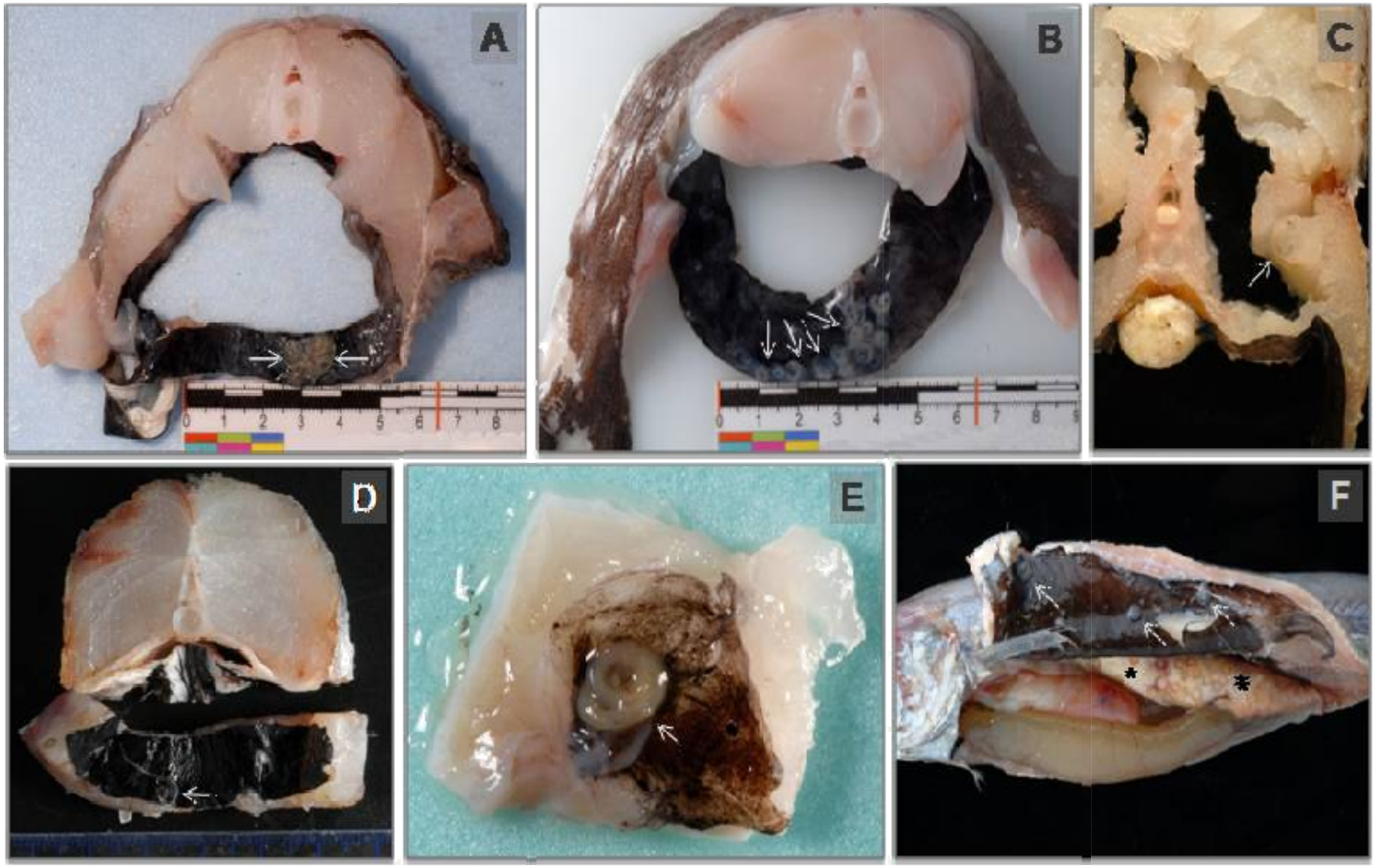
A
B
C
D
E
F

Figure 9.2. (A-F). Transversal sections of *Lophius budegassa* **(A, B)**, *Macrourus berglax* **(C)**, *Merluccius merluccius* **(D)**, *Sebastes mentella* **(E)**, and an individual of *Micromesistius poutassou* **(F)** showing higher amounts of anisakids at the hypaxial region than at the epaxial musculature. Parasites are located encysted inside the belly flaps and muscle, as well as covering them. F also shows a high quantity of embedded worms in some internal organs such as the liver (black asterisks). White arrows: Anisakid larvae.

Table 9.2. Fish species studied, including the total number of individuals dissected (N), total muscular parasitized fishes from each lot and the individuals selected for parasite sequencing, total muscular larvae found in the selected fishes and the site of infection in the hosts, and anisakids (species and number) diagnosed after sequencing, with their corresponding GenBank accession numbers.

Fish species (N)	Parasitized hosts/Selected hosts for parasite sequencing	Total count of parasites in selected hosts	Host-site of infection		Parasites successfully sequenced	Etiologic agents (parasite species diagnosed and number)	GenBank accession number
			Hypaxial	Epaxial			
Macrourus berglax (50)	20/10	43	43	0	11	*Anisakis simplex* sensu stricto (11)	KF512829 - KF512839
Macruronus magellanicus (17)	16/2	5	5	0	2	*Anisakis pegreffii* (2)	KF512840, KF512841
Micromesistius poutassou NEAFC (50)	41/9	74	72	2	9	*Anisakis simplex* sensu stricto (9)	KF512842 - KF512850
Coryphaenoides rupestris (50)	6/1	1	1	0	1	*Anisakis simplex* sensu stricto (1)	KF512857
Sebastes mentella (50)	29/3	59	59	0	3	*Anisakis simplex* sensu stricto (3)	KF512858 - KF512860
Micromesistius poutassou ICES (329)	271/10	60	49	11	10	*Anisakis simplex* sensu stricto (4)	KF512861 - KF512864
						Anisakis pegreffii (6)	KF512851 - KF512856
Scomber scombrus (236)	84/2	4	4	0	3	*Anisakis simplex* sensu stricto (1)	KF512865
						Pseudoterranova sp. (2)	KF512907, KF512908
Lepidorhombus whiffiagonis (50)	18/3	6	4	2	3	*Anisakis simplex* sensu stricto (3)	KF512866 - KF512868
Lophius budegassa (50)	46/14	557	539	18	15	*Anisakis simplex* sensu stricto (12)	KF512869 - KF512880
						Pseudoterranova sp. (3)	KF512909 - KF512911
Lophius piscatorius (50)	36/10	52	52	0	10	*Anisakis simplex* sensu stricto (10)	KF512881 - KF512890
Merluccius merluccius (50)	45/15	1994	1970	24	18	*Anisakis simplex* sensu stricto (16)	KF512891 - KF512906
						Pseudoterranova sp. (2)	KF512912, KF512913

NEAFC, North East Atlantic Fisheries Commission; ICES, International Council for the Exploration of the Sea.

9.4. DISCUSSION

The European fish industry complies with the current legislation, recommended practices, and guidelines implemented by the governments and regulatory agencies, to carry out parasite controls on their facilities and products. Basically, official inspections and self-management programs based on the HACCP system comprise the current practices to eliminate or reduce the risk of this biological hazard in seafood products. Despite this, there is still a historical concern regarding consumer complaints or lawsuits in trade operations when a contaminated fish lot reaches any given susceptible step from the sea to the plate. These problems arise above all due to the absence of an established legal maximum limit for anisakids in fish lots. Specifically, Regulation EC 178/2002 states that food shall not be placed on the market if it is unsafe (i.e., injurious to health or unfit for human consumption). Regardless of the treatments that could be applied on parasitized fishes to prevent the ingestion of viable parasites (i.e, zoonoses), any parasitized fish is unfit for reasons of contamination by extraneous matter or otherwise. Moreover, the subjective application of some confusing concepts such as ‘‘visible parasite’’ and ‘‘clearly contaminated,’’ specified in
the European Hygiene Package (2004), Council Regulation (EC) 2406/96, and Commission Regulations (EC) 1662-1664/2006, makes it possible that each operator follows its own rules. In fact, the absence of a criterion standard method and the lack of an analytical critical limit to distinguish an acceptable from an unacceptable infected fish lot provoke a heterogeneous *modus operandi* at self-management controls. This circumstance leads to multiple methods of managing parasitized fish lots and does not prevent rejections in the last points of fish value chain due to visually highly parasitized fish. This is the reason why in the absence of an inspection standard and a “*quantum satis”* statement for parasites, it is important that fish industries embrace a common language to operate (i.e., standard terminology) that guarantees inspectors and consumers an appropriate predictive scoring of parasitized fish.

SADE scores can be fitted to any commercial fish lot from a particular fishing ground, size-maturity-age of fish, fish cohort, or postharvest condition. This information could then be used to propose risk mitigation and prevention measures at harvesting, processing, and postprocessing. Moreover, SADE scoring is an added value tool that improves the *modus operandi* at self-management processes by increasing (1) consumer, professional, and trade confidence (due to a standardized working method); and (2) competitive strengthening in fish operators by achieving a higher standard quality and preventing product losses. In fact, SADE may accurately predict outcomes for the fishery industries related to the unaesthetic images that significantly impact on the commercial value of the affected products. This fact has been forcing the seafood industry to discard large quantities of fish and to intensify quality-inspection protocols on seafood products.

Thereafter, the SADE scoring system can be adapted or modified as needed over time. The SADE lexicon could be multifold by adding variables (i.e., diagnostic factors) into subcategories. This illustrates the future increasing complexity of stage grouping, when factors other than S, A, D, and E, such as branches and

leaves, are included and added to the main tree trunk. SADE was constructed to assess four basic indicative categories, but this nodal staging system can be adapted to build more ‘‘look-up’’ predictive classifications in other well-known muscular parasites in seafood products. Therefore, scoring would give a common language for evaluating parasite risk in fish inspections, becoming a technological tool operating *in silico* for research, industrial, and commercial use within HACCP programs. Scoring is also useful in harmonization and prospection of research results derived from large data sets and from the peer-reviewed literature (e.g., meta-analysis). In this way, the SADE system has been constructed as a ‘‘bin model.’’ That means that it can use the diagnosis of an infected fish lot already in the bin (i.e., in a given subcategory) to predict what will happen to a new fish lot placed in that bin.

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CHAPTER 10

Transfer of knowledge

PARCODE: An innovate tool for parasite management

10.1. INTRODUCTION

PARCODE is a web platform with a stable operating structure and a translational approach to the fishing sector which is addressed to companies, organizations, and institutions involved in the fishing activity or research, as well as to consumers.

"About us"

The "PARCODE" idea arised from researchers of the group of Marine Ecology and Biodiversity (ECOBIOMAR), which belongs to the department of Resources and Marine Ecology, Institute of Marine Research (IIM-CSIC), in Vigo (Spain).

"Contact"

After several years of research in the field of fish parasites, numerous reasons drove the creation of this online platform:

- a high amount of interesting, useful and valuable results generated after all experimental studies developed in this thesis,
- the accessible but very scattered background of information existing on this matter, which should be included into strategic plans for quality management of the companies,
- the urgent need of companies to provide answers to a growing number of problems concerning parasites in fishery products,
- the legal ambiguity which drives public health official inspectors to a lack of consensus in their professional practice,
- a largely uncoordinated situation at the scientific level,
- consummers' increasing interest and demand for information on key issues concerning health, nutrition and food quality, such as parasites or zoonoses,
- a spirit of innovation and our ability to convert scientific developments into knowledge with high added value.

Although the main purpose of PARCODE is to disseminate scientific and technical knowledge which have been learned from many years of research among the network users, one of the most important points arising in this context is that information is not being transmitted in a context beyond the experience of the target interlocutor, nor using an appropriate language. As FAO cited in 2002 in its document on Food Quality and Safety Systems, "transmitting or talking over the receiver's head", as it is usually called, is for example, to transmit detailed and profound scientific messages to a receiver without a scientific background.

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have been working for several decades, in collaboration with national governments, scientific institutions, the food industry, consumers and others, to improve the safety and quality of food. Food safety officials in various contexts have established new communication forums that bring industry, consumer representatives and government officials together to discuss problems, priorities and strategies in collegial, non-adversarial settings. As the FAO and the WHO mentioned in the document "Food safety risk analysis" (2007), while there will probably always be a need for written documents, scientific reports and official government analyses of food safety matters, effective communication often requires some "non-meeting"

additional approaches, which can be quite creative. For instance, a workshop on scientific and economic aspects of the food safety controls would be likely to attract robust food industry participation, while a panel discussion on the latest advances in risk analysis methodologies should appeal to many academic experts. That is the thinking behind PARCODE platform has been created.

10.1.1. Official inspection bodies and scientific community

Concerning the official inspection bodies, the official veterinarian and official auxiliaries are to maintain up-to-date knowledge and to keep abreast of new developments through regular continuing education activities and professional literature (Point A.5 and B.6 of Chapter IV of Regulation (EC) 854/2004). Specifically, as Real Decreto 1614/2008 from the Spanish law states in Annex VII, the personnel involved at all stages of the food chain concerning acquatic animals must be regularly involved in trainning in clinical signs, epidemiological investigation and control of epizootic diseases in alert exercises in real time. Via PARCODE it is intended to offer both official inspection staff and scientists in the field, as many regular updates on the latest scientific developments as possible. PARCODE will regularly also announce all trainning activities for professionals that may be taken place.

10.1.2. Fishing industry

As Commission Decision 94/356/EEC specifies in Pt. 2 of Art. 1, establishments such as fishing industries, may use guides of good manufacturing practice drawn up by appropriate professional organizations and acceptable to the competent authorities. In point 1 of Chapter I in Annex, this document also recommends enterprises to be assisted if necessary by specialists who will help to solve difficulties as regards assessment and control of critical points. Those types of quality control specialists may include experts on biological hazards among others, connected with a particular product group. Processors must have scientific information on potential hazards associated with raw material and products for further processing (Codex Alimentarius, CAC/RCP 52-2003). Where expertise is not available in the establishment, it should be obtained from external sources.

Such types of documents as guides of good manufacturing practices, as well as external specialized assessment on fish parasites already exist and PARCODE make it available to the platform's users.

10.1.3. Consumers

When one of the goals is to engage and inform to a non-specialist public, messages need to be presented in media the audiences pay attention to. Internet discussion boards, chat rooms, and other gatherings such as those proposed by PARCODE, enable participants to share views and concerns and to obtain valuable information. In general, large public meetings are not especially effective for eliciting the transparent dialogue that risk communication seeks to achieve.

Moreover, as the White Paper of Food Safety (1999) stated in Chapter 7, in all aspects related to food safety, risk communication should not be a passive transmission of information, but should be interactive, involving a dialogue with and feedback from all stakeholders. Thus it is essential that consumers, as recognised stakeholders, must be taken into account by providing a framework for discussions (public hearings) between them and scientific experts.

From the outset of this project, a series of major partners and participating entities as the Administration, enterprises, consulting companies, universities, R&D&I platforms or other research centers, have given their support and have shown their readiness to help.

"Partners directory"

PARCODE's main objectives are on the one hand to avoid the health risks that might result from the consumption of fish parasitized, and secondly to prevent situations of commercial rejection in certain fish batches containing from excessive to visible parasitism. Therefore, this platform has been created in order to try to advise and provide practical solutions, as well as relevant information to the problems inherent in managing stocks, fishery products and by-products infected with parasites, especially those with allergenic potential zoonotic relevance and/or negative effects on commercial quality.

10.2. MATERIALS AND METHODS

The creation of the website was carried out by a specialized company (www.reinografico.es), that featured the work guided and coordinated by ECOBIOMAR's researchers.

PARCODE has been created by using Flash with Action Script design/programming, which enables the website to have stability and a wide range of action. Through the use of Unicode, there is no need to create the underlying programming for each language separately. The correct use of Flash offers outstanding advantages as:

- Safety contents
- Programming versatility
- Fast software download and updates
- Adaptability to full screen size without losing resolution
- Easy content dissemination due to its capability to be exported to different predetemined formats
- Video display without plugings
- Compatibility with 97 per cent of browsers and all the operating systems. The web design company is currently working on the development of alternatives for Android and Ipad.

PARCODE website has been divided in two principal areas:

- Main menu including: “Conócenos” (“About us”), “Directorio de socios” (“Partners directory”), “Centro de documentación” (“Documentation centre”), “PARCODE Campus”, “Buzón de sugerencias” (“Suggestions mailbox”), “Enlaces de interés” (“Links of interest”), “Asóciate” (“Join now”) and “Contacto” (“Contact”).
- “Servicios” (“Services”) area including: “Asesora” (“Advice”), “Actualiza” (“Updating”), “Visualiza” (“Visualization”) and “Normaliza” (“Standardisation”).

As previously mentioned, a large amount of accessible but very scattered information existing on food safety and quality, and more specifically on seafood managing, was organized, centralised and classified becoming the “Documentation centre”. Furthermore, all results obtained from the investigations on which this dissertation is based, have been selected, transformed and adapted from an extensive epidemiological database into an easily understandable format for all potential users. They are shown in “Results” of this chapter and they will shortly be incorporated into the platform as basis for the creation of dynamic risk maps; the services’ ultimate aim offered in “Advice”.

One strategy that was used to publicize the website was the organization of: (1) a symposium, (2) a R&D&I forum, and (3) a series of round tables. The purpose of the three meetings was to discuss the food biosecurity and management of parasitism in fishery products, from a scientific-technical point of view and directly with professionals of the fishing industry. Specifically, roundtables aimed at addressing the problem

of parasites in commercial fisheries, from three different perspectives; industry, scientific community and official inspection bodies.

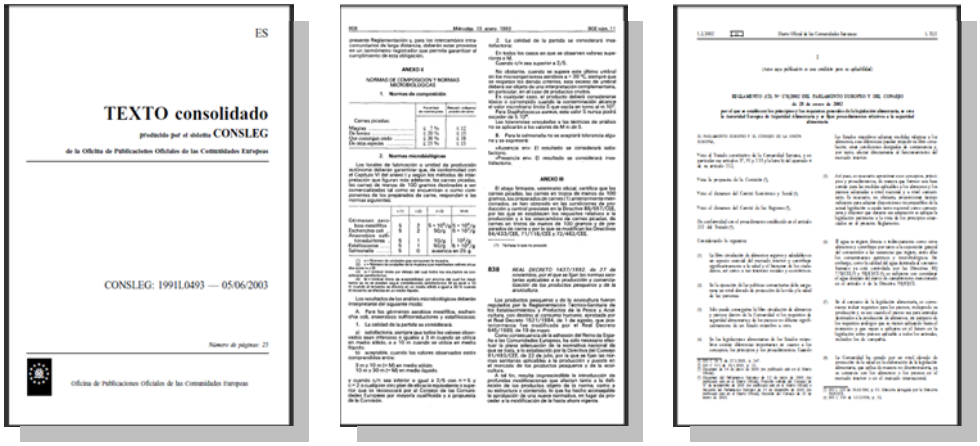
10.3. RESULTS

Apart from the website buttons such as “About us”, “Partners directory” and “Contact”, previously referred to in the Introduction of this chapter, the remaining tabs incorporated in the main menu including “Documentation centre”, “PARCODE Campus”, “Suggestions mailbox”, “Links of interest” and “Join now”, are described and illustrated below:

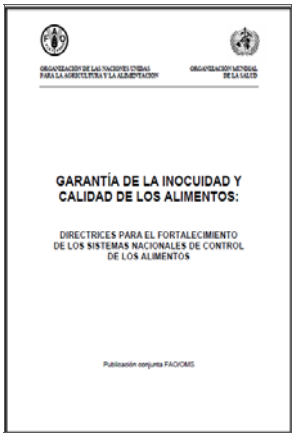
“Documentation centre”:

Relevant and public scientific, technical and policy-related documents concerning food safety and quality, and more specifically regarding the management of fishery products, have been included in the “Documentation centre”, classified and organized as follows:

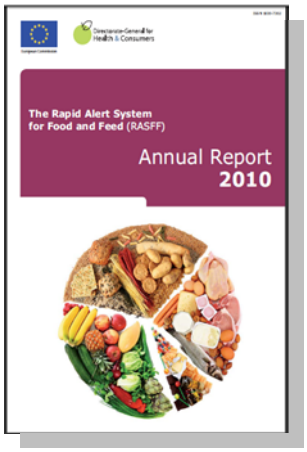
- Texts related to legislative aspects



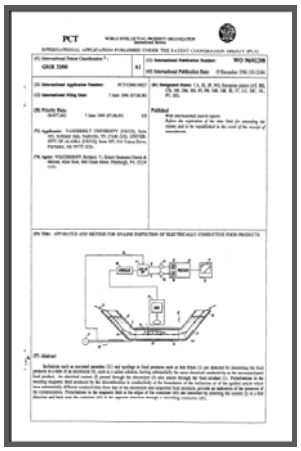
- Documents dealing with official recommendations

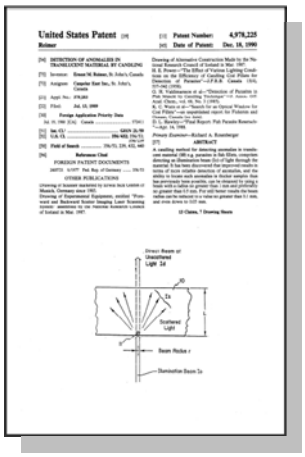


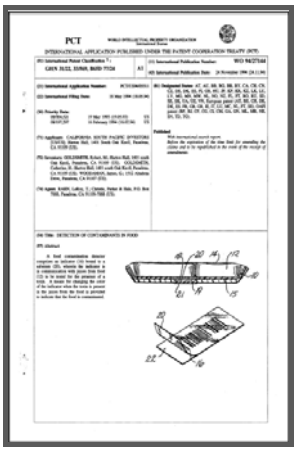
- White papers and some scientific-technological manuscripts



- Technological developments.

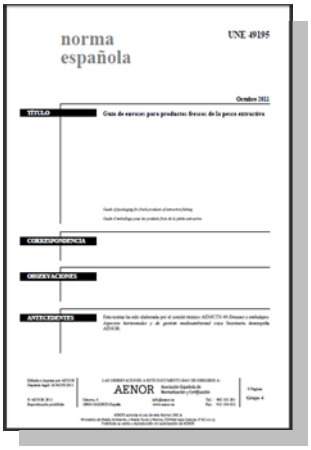


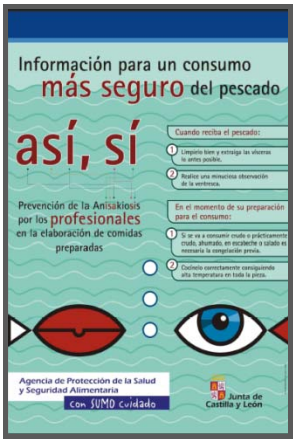




***"PARCODE Campus"*:**

This is an area which includes training materials such as manuals and guides for fishing company workers closely related to the tasks of management, production, or processing seafood products. The material herein proposed aims to contribute to the improvement of food safety and hygiene practices during fishing, handling, processing, storaging or selling fishery products.





"Suggestions mailbox":

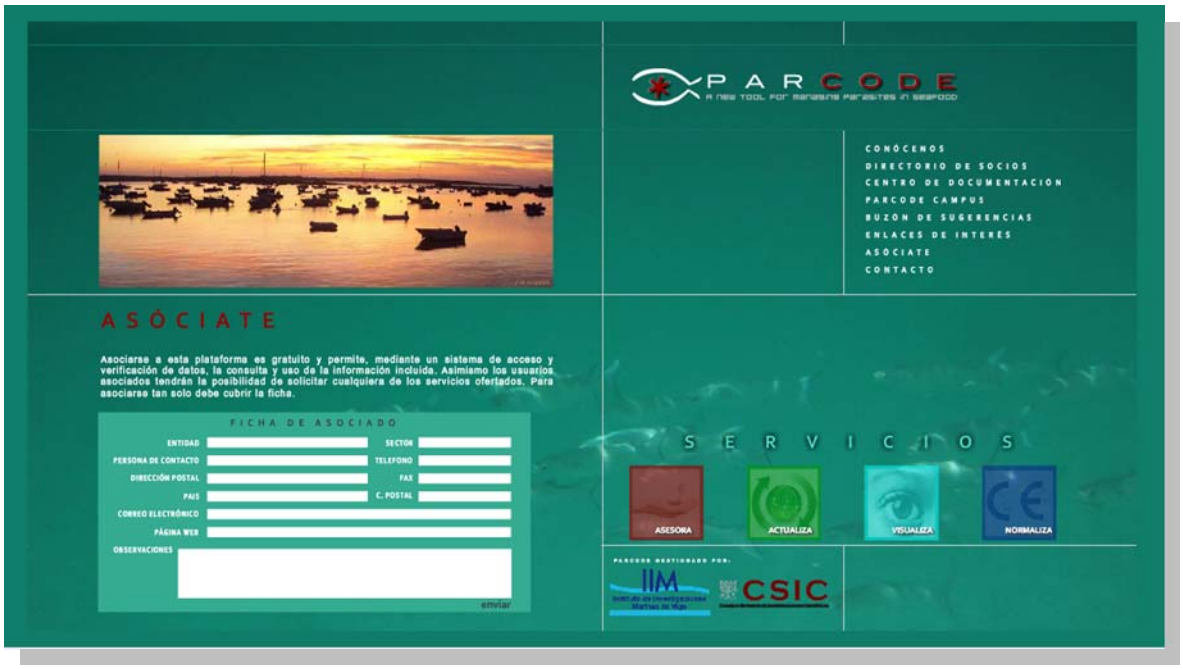
The "Suggestions mailbox" allows PARCODE platform receiving objections and comments as well as getting closer to the needs of users and partners by knowing their points of view.

"Links of interest":

This section offers helpful links to websites, news and publications concerning the fishing sector to all users.

"Join now":

One of the claims used to invigorate the PARCODE platform was to provide users the opportunity to become a partner for free, by obtaining as benefit the possibility to enter into the restricted areas.



PARCODE was raised with the idea to disseminate the important quantity of valuable, helpful and profitable results generated after the scientific research programme considered in this thesis.

All the raw results obtained were filtered, intensively worked and converted into a format easily assimilated by all potential users. Integrating documentary, technical, scientific and graphic information into a single easily comprehensible and accessible sheet for each fish species was the challenge. Each sheet, edited in PDF format includes data about:

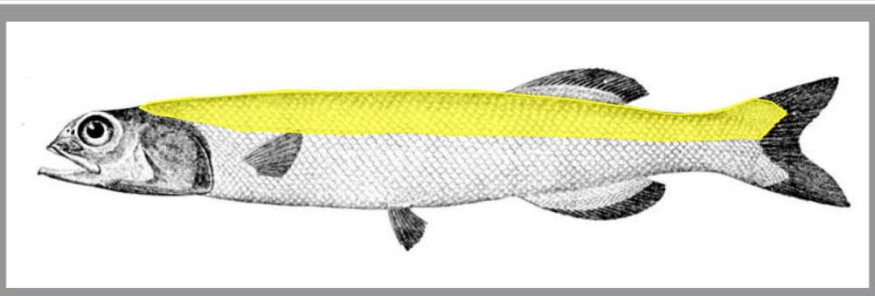
- Fish stock/samples (form of presentation and origin)
- Parasite/s (etiological agent, relevant information, prevalence and type of parasitic implications)
- Risk management measures (critical control points and corrective actions)

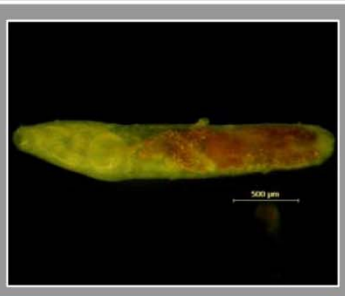
A total of 13 sheets have been created as a practical application of the results of this dissertation, also included in the Project EPISTOCK (INCITE-44.02.741A.771.0, Xunta de Galicia). By the moment, they are exclusively created in Spanish version for the following 13 fish species:

- *Alepocephalus bairdii*
- *Coryphaenoides rupestris*
- *Lepidorhombus whiffiagonis*
- *Lophius budegassa*
- *Lophius piscatorius*
- *Macrourus berglax*
- *Macruronus magellanicus*
- *Merluccius merluccius*
- *Merluccius polli*
- *Micromesistius poutassou* (ICES)
- *Micromesistius poutassou* (NEAFC)
- *Scomber scombrus*
- *Sebastes mentella*

Alepocephalus bairdii

Trematodiasis





LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. XII, 56°49'N 19°28'W.

PARÁSITO:

- Agente etiológico: *Lecithophyllum botryophorum.*

- **Información de interés:** A pesar del pequeño tamaño que presentan, su color oscuro hace que sean fácilmente visibles.

- **Prevalencia:** moderada (20-50%).

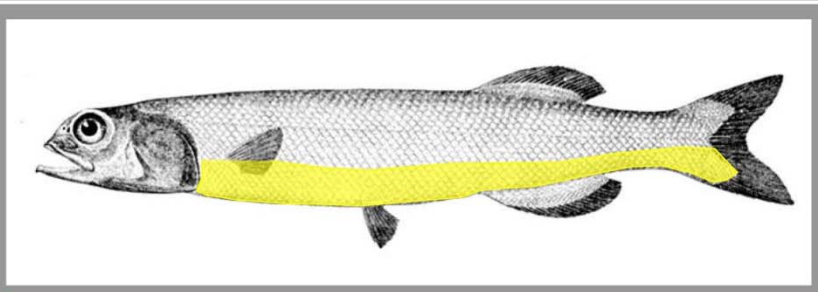
- **Tipo de afección:** calidad comercial.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** se localizan especialmente en la musculatura dorsal del tórax, extendiéndose hacia la musculatura lumbar y el pedúnculo caudal.

- **Medidas Correctoras:** como medida preventiva y ante problemas de rechazo comercial se recomienda eliminar la musculatura epiaxial de la región torácica (claramente la más afectada).

Trematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. XII, 56°49'N 19°28'W.

PARÁSITO:

- **Agente etiológico:** *Steningophorus margolis.*

- **Información de interés:** Se encuentra en los peces con mayores rangos de calibre.

- **Prevalencia:** accidental (<10%).

- **Tipo de afección:** calidad comercial.

MEDIDAS DE GESTIÓN:

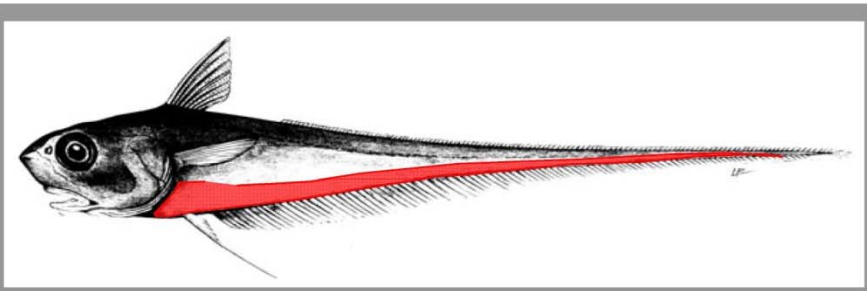
- **Puntos de Control Crítico:** normalmente se aloja en la musculatura que limita la cavidad perivisceral.

- **Medidas Correctoras:** debería considerarse como medida profiláctica el chequeo de la musculatura perivisceral e hipoaxial torácica. Asimismo se recomienda eliminarla en el momento de eviscerar (para evitar migraciones del parásito desde la zona afectada al resto de la musculatura.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Coryphaenoides rupestris

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. XII, 58°38'N 19°03'W.

PARÁSITO:

- Agente etiológico: *Anisakis simplex.*

- **Información de interés:** tan sólo el 6% de los individuos estudiados estaban afectados.

- **Prevalencia:** accidental (<10%).

- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

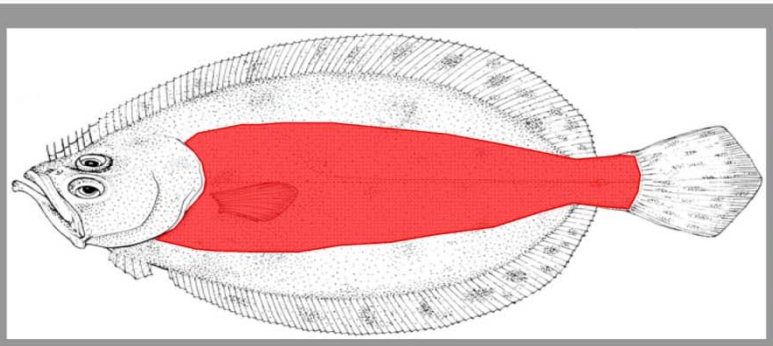
- **Puntos de Control Crítico:** parásitos bastante localizados, en el techo de la cavidad perivisceral.

-**Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Lepidorhombus whiffiagonis

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. VIIh (Gran Sol 25-26/E1), 48°25'N 08°53'W.

PARÁSITO:

- Agente etiológico: *Anisakis simplex.*

- **Información de interés:** no se encontró relación entre el calibre del pez y el número de parásitos.

- **Prevalencia:** moderada (20-50%).

- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

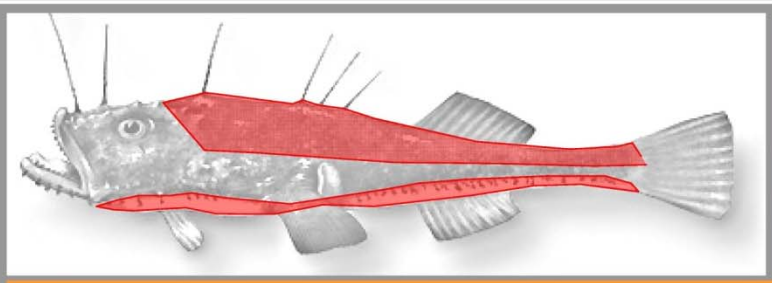
- **Puntos de Control Crítico:** localizados en la musculatura torácica, tanto a nivel dorsal como ventral, y circundando a la espina dorsal.

- **Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Lophius budegassa

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. VIIj (26/D9), 48°56'N 10°11'W.

PARÁSITO:

- Agente etiológico: *Anisakis simplex/Pseudoterranova decipiens.*

- **Información de interés:** prácticamente la totalidad de peces estudiados se encontraban afectados por este parásito.

- **Prevalencia:** alta (>50%).

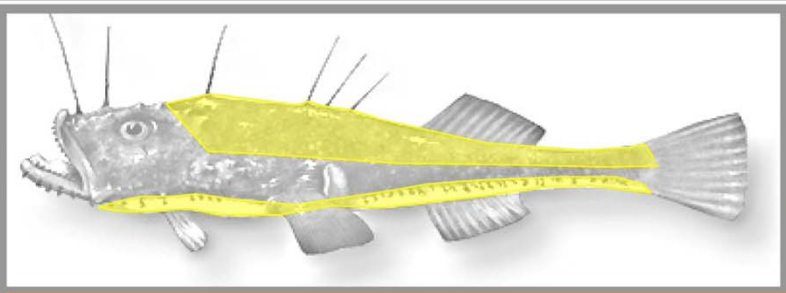
- **Tipo de afección:** calidad higiénico-sanitaria.

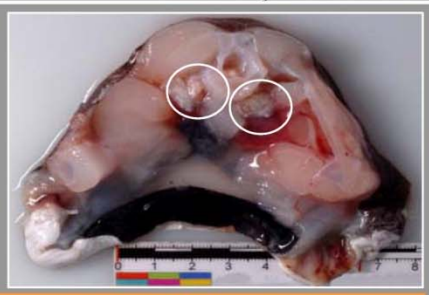
MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** *Anisakis simplex* localizados especialmente en la zona perivisceral ventral, y presentando una densidad elevada (31 larvas/Kg). *Pseudoterranova decipiens* con mucha menor prevalencia y densidad de infección, alojados tanto dorsal y superficialmente en la región torácica, como por toda la musculatura de la zona lumbar.

-**Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Microsporidiosis





LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. VIIj (26/D9), 48°56'N 10°11'W.

PARÁSITO:

- **Agente etiológico:** *Spraguea lophii.*

- **Información de interés:** los xenomas o sacos que forman estos microsporidios tienen la forma de pequeñas esferas blancas que se agrupan en unas estructuras fácilmente detectables.

- **Prevalencia:** moderada (20-50%).

- **Tipo de afección:** calidad comercial.

MEDIDAS DE GESTIÓN:

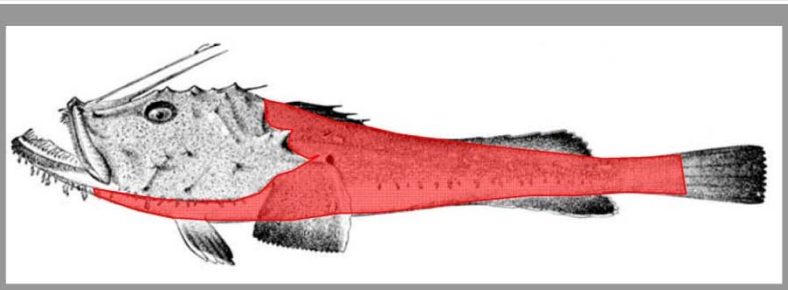
- **Puntos de Control Crítico: se encuentran** casi exclusivamente adheridos al periostio de las vértebras cervicales, torácicas y lumbares, con una intensidad de infección mayor en las zonas cervical y torácica.

- **Medidas Correctoras:** las formas de presentación de este parásito son lo suficientemente visibles como para ocasionar rechazo comercial. Se recomienda eliminar las estructuras o zonas más parasitadas.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Lophius piscatorius

Nematodiasis



LOTE:

- **Modo de presentación:** congelado/entero.
- **Procedencia:** FAO 27 (ICES), Sub. VIIh (Mar Céltico), 48°25'N 09°21'W.

PARÁSITO:

- **Agente etiológico:** *Anisakis simplex.*
- **Información de interés:** 2 de cada 3 individuos estudiados estaban afectados por este parásito.
- **Prevalencia:** alta (>50%).
- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

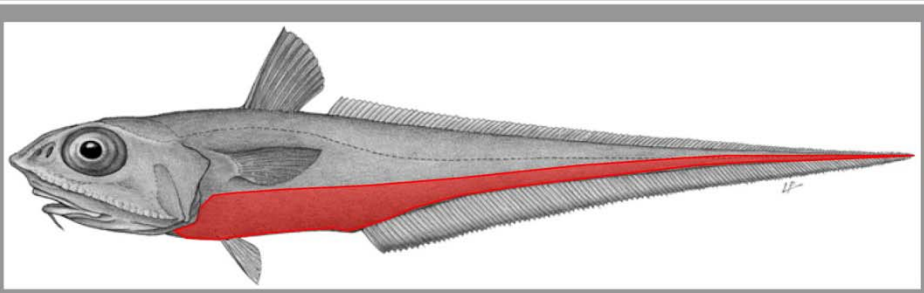
- **Puntos de Control Crítico:** localizados especialmente en la zona perivisceral ventral, pero también presentes en el tejido subcutáneo de la zona dorsolateral del tórax, y junto a vértebras torácicas.

-**Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Macrourus berglax

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 21 (NAFO), 48°38'N 45°45'W.

PARÁSITO:

- **Agente etiológico:** *Anisakis simplex.*

- **Información de interés:** no se encontró relación entre el calibre del pez y el número de parásitos.

- **Prevalencia:** moderada (20-50%).

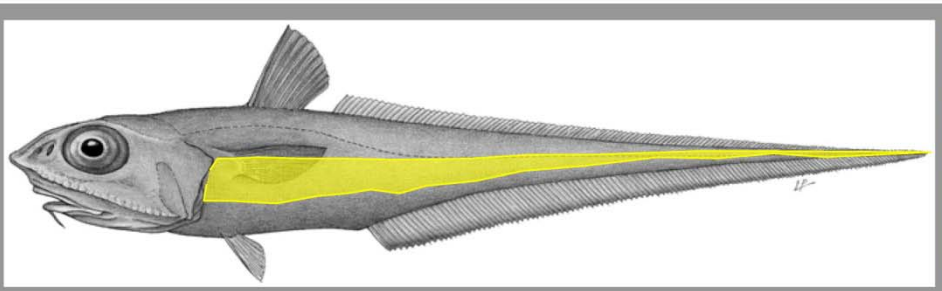
- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** los anisákidos se encuentran localizados en el suelo torácico de la cavidad perivisceral.

-**Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Acantocefaliasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 21 (NAFO), 48°38'N 45°45'W.

PARÁSITO:

- **Agente etiológico:** *Echinorhynchus gadi.*

- **Información de interés:** en todos los casos este parásito se situaba muy superficialmente en el pez.

- **Prevalencia:** baja (10-20%).

- **Tipo de afección:** calidad comercial.

MEDIDAS DE GESTIÓN:

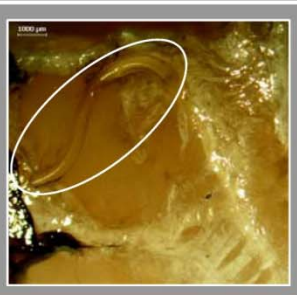
- **Puntos de Control Crítico:** están normalmente localizados en las capas musculares laterales de la cavidad perivisceral, y se encuentran considerablemente cerca de la epidermis (a menos de 3 mm).

- **Medidas Correctoras:** se recomendaría como medida profiláctica, además de eviscerar en la menor brevedad posible tras la captura, eliminar la musculatura perivisceral del animal durante dicho proceso para evitar migraciones desde dicha zona al resto del pescado.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Macruronus magellanicus

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 41 (Malvinas), 52°29'S 62°12'W.

PARÁSITO:

- Agente etiológico: *Anisakis pegreffii.*

- **Información de interés:** no se encontró relación entre el calibre del pez y el número de parásitos.

- **Prevalencia:** moderada (20-50%).

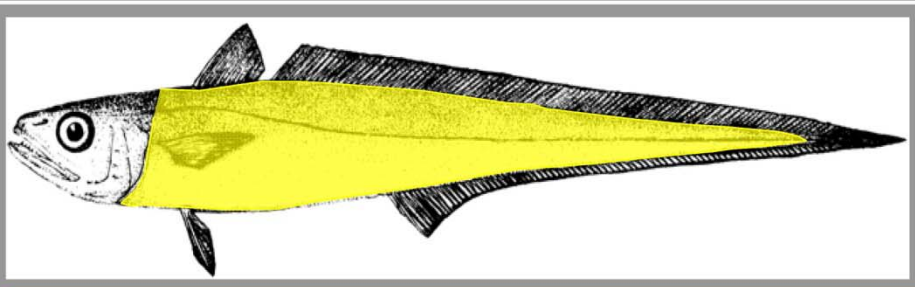
- **Tipo de afección:** calidad higiénico-sanitaria.

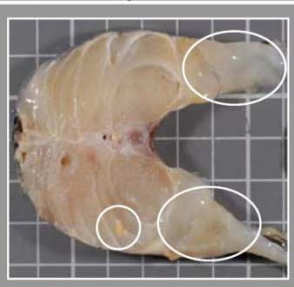
MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** parásitos localizados sobre todo en la musculatura perivisceral, y en menor medida junto a la espina dorsal en la región torácica.

-**Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Mixosporidiosis





LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 41 (Malvinas), 52°29'S 62°12'W.

PARÁSITO:

- **Agente etiológico:** *Kudoa* spp.

- **Información de interés:** el parásito puede manifestarse en forma de quistes de color mostaza o provocando autolisis (aspecto lechoso) en el músculo.

- **Prevalencia:** alta (>50%).

- **Tipo de afección:** calidad comercial.

MEDIDAS DE GESTIÓN:

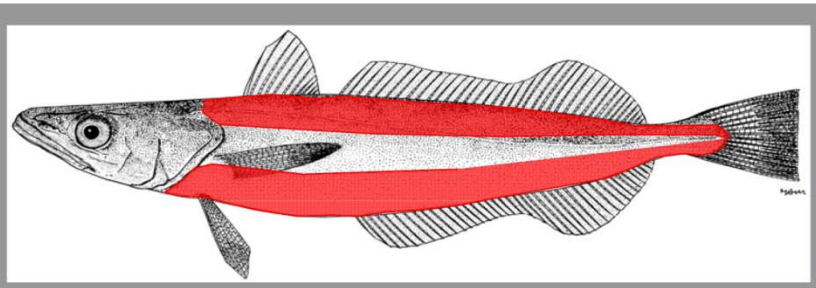
- **Puntos de Control Crítico:** localizado muy disperso por toda la musculatura.

- **Medidas Correctoras:** las formas de presentación de este parásito son lo suficientemente visibles como para ocasionar rechazo comercial. Se recomienda eliminar las zonas más parasitadas.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Merluccius merluccius

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. VIIc (35/D7), 53°25'N 12°20'W.

PARÁSITO:

- Agente etiológico: *Anisakis simplex/Pseudoterranova decipiens.*

- **Información de interés:** prácticamente la totalidad de peces estudiados se encontraban afectados por este parásito.

- **Prevalencia:** alta (>50%).

- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** están localizados especialmente en la zona perivisceral ventral, lateral y dorsal (en menor proporción), pero también presentes en la musculatura lumbar ventral y junto a vértebras torácicas. Esta parasitación presenta una densidad extremadamente elevada (254 larvas/Kg).

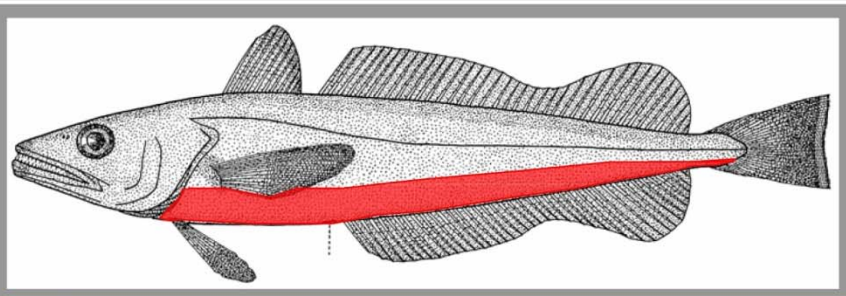
-**Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

A la hora de diseñar una medida profiláctica, la gran dispersión que este parásito presenta hace difícil el poder resaltar una zona especialmente contaminada. La elevadísima prevalencia y densidad de infección hacen que *Merluccius merluccius* sea una especie susceptible de ocasionar problemas durante su comercialización y consumo. Además de las posibles repercusiones que este parásito pudiera ocasionar a nivel sanitario, la citada presencia de tal cantidad de nematodos podría afectar a la calidad visual, generando rechazo en el consumidor.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Merluccius polli

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 47 (Angola), 8°56'S 12°48'E.

PARÁSITO:

- Agente etiológico: *Anisakis spp.*

- **Información de interés:** menos del 6% de los individuos estudiados estaban afectados.

- **Prevalencia:** accidental (<10%).

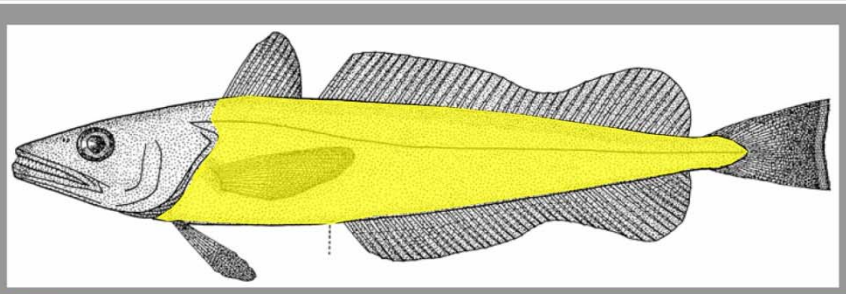
- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** localizado en la musculatura de la cavidad perivisceral y en la circundante a la espina dorsal.

- **Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Trematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 47 (Angola), 8°56'S 12°48'E.

PARÁSITO:

- **Agente etiológico:** *Stephanostomum cestillium.*

- **Información de interés:** A pesar de su baja prevalencia, se encuentra muy disperso por toda la musculatura.

- **Prevalencia:** baja (10-20%).

- **Tipo de afección:** calidad comercial.

MEDIDAS DE GESTIÓN:

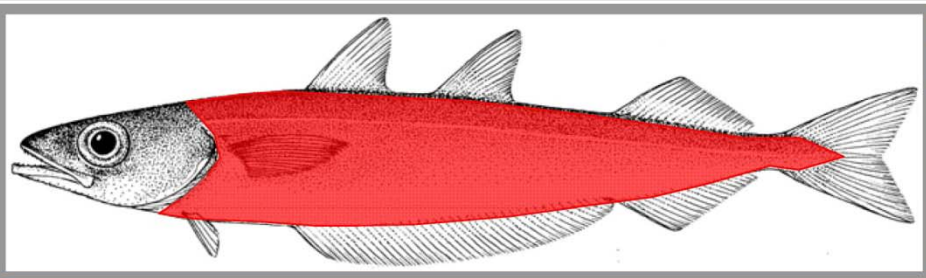
- **Puntos de Control Crítico:** localizado muy disperso y considerablemente cerca de la epidermis (<5mm).

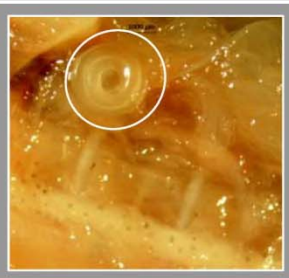
- **Medidas Correctoras:** se aconseja someter al pescado a unos procesos previos y controlados de congelación y cocinado. Ante la duda de un rechazo comercial, se recomienda eliminar las zonas visiblemente parasitadas.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Micromesistius poutassou

Nematodiasis





LOTE:

- **Modo de presentación:** fresco/entero.
- **Procedencia:** FAO 27 (ICES), 42°33'N 08°59'W.

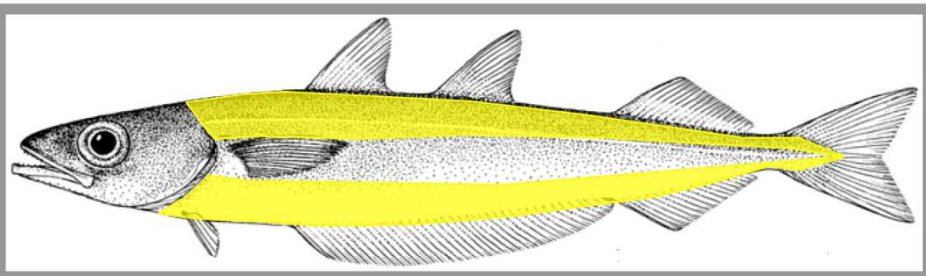
PARÁSITO:

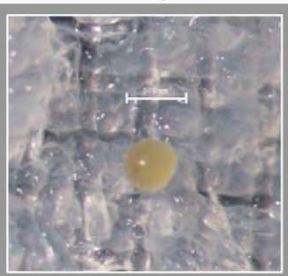
- **Agente etiológico:** *Anisakis simplex/Anisakis pegreffii.*
- **Información de interés:** no se encontró relación entre el calibre del pez y el número de parásitos.
- **Prevalencia:** alta (>50%).
- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** es encuentran distribuidos por todo el pescado presentando alta densidad, y muy especialmente por la musculatura ventral.
- **Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Acantocefaliasis





LOTE:

- **Modo de presentación:** fresco/entero.
- **Procedencia:** FAO 27 (ICES), 42°33'N 08°59'W.

PARÁSITO:

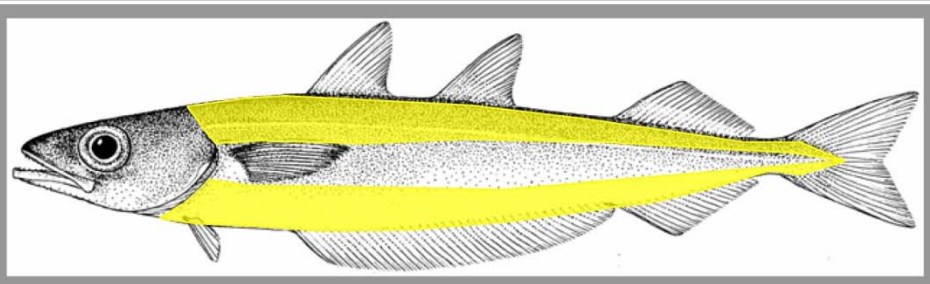
- **Agente etiológico:** *Echinorhynchus gadi.*
- **Información de interés:** A pesar de su pequeño tamaño, la elevada prevalencia y la enorme dispersión, hacen que sean fácilmente visibles.
- **Prevalencia:** alta (>50%).
- **Tipo de afección:** calidad comercial.

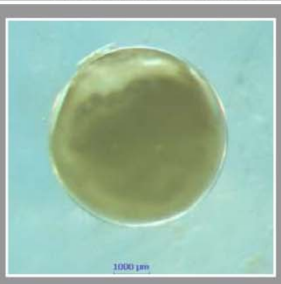
MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** Se localizan uniformemente por toda la musculatura dorsal, y en menor medida por la ventral.
- **Medidas Correctoras:** se aconseja someter al pescado a unos procesos previos y controlados de congelación y cocinado. Ante la duda de un rechazo comercial, se recomienda eliminar las zonas visiblemente parasitadas.

Micromesistius poutassou

Trematodiasis





LOTE:

- **Modo de presentación:** fresco/entero.
- **Procedencia:** FAO 27 (ICES), 42°33'N 08°59'W.

PARÁSITO:

- **Agente etiológico:** *Stephanostomum cestillium.*
- **Información de interés:** Estos parásitos de forma esférica y color pálido presentan baja intensidad de infección.
- **Prevalencia:** moderada (20-50%).
- **Tipo de afección:** calidad comercial.

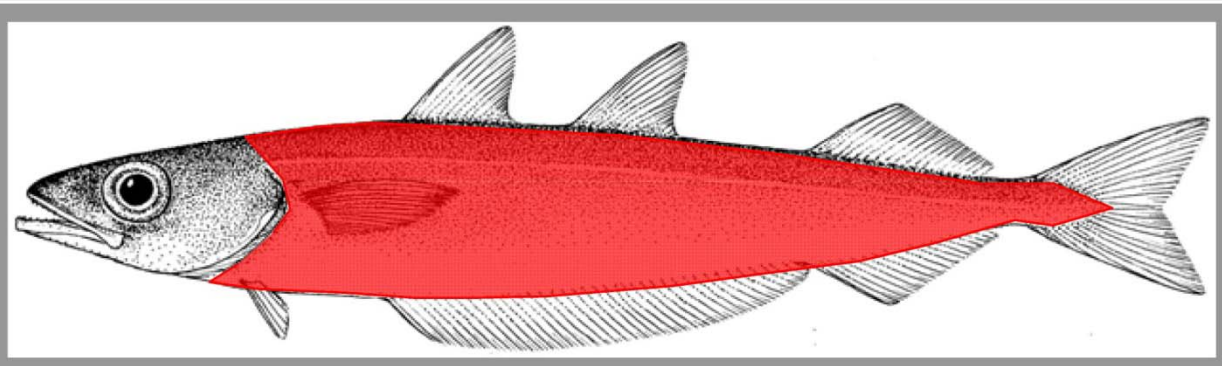
MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** se distribuyen tanto por la musculatura dorsal como por la ventral, y especialmente en la musculatura más ventral de la cavidad perivisceral.
- **Medidas Correctoras:** en casos de mayor intensidad de infección o para prevenir rechazo comercial debido a su tamaño, se recomendaría quitar, conjuntamente con las vísceras, la musculatura ventral que conforma la cavidad perivisceral.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Micromesistius poutassou

Nematodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (NEAFC), Sub. VIb, 58°34'N 15°04'W.

PARÁSITO:

- Agente etiológico: *Anisakis simplex.*

- **Información de interés:** 4 de cada 5 individuos estudiados estaban afectados por este parásito, presentando además, una elevada densidad de infección.

- **Prevalencia:** alta (>50%).

- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** parásitos localizados muy dispersos por toda la musculatura.

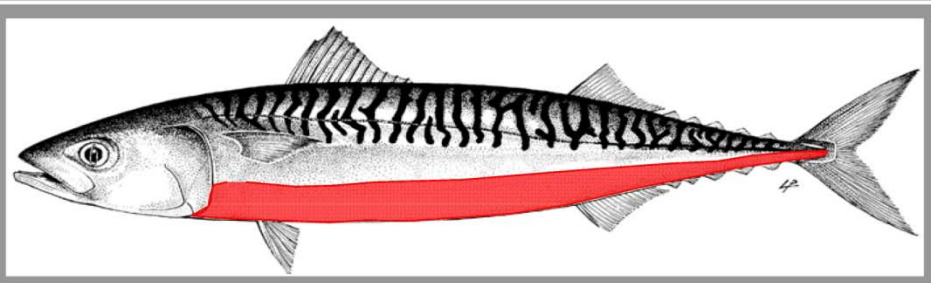
-**Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

La elevada prevalencia y densidad de infección (muy superior a la máxima recomendada por el CODEX ALIMENTARIUS), hacen que *Micromesistius poutassou* sea una especie susceptible de ocasionar problemas durante su comercialización y consumo. Además de las posibles repercusiones que este parásito pudiera ocasionar a nivel sanitario, la citada presencia de tal cantidad de nematodos podría afectar a la calidad visual, generando rechazo en el consumidor.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Scomber scombrus

Nematodiasis



LOTE:

- Modo de presentación: fresco/entero.

- Procedencia: FAO 27 (ICES), 43°58'N 07°12'W.

PARÁSITO:

- **Agente etiológico:** *Anisakis spp./Pseudoterranova decipiens.*

- **Información de interés:** no se encontró relación entre el calibre del pez y el número de parásitos.

- **Prevalencia:** moderada (20-50%).

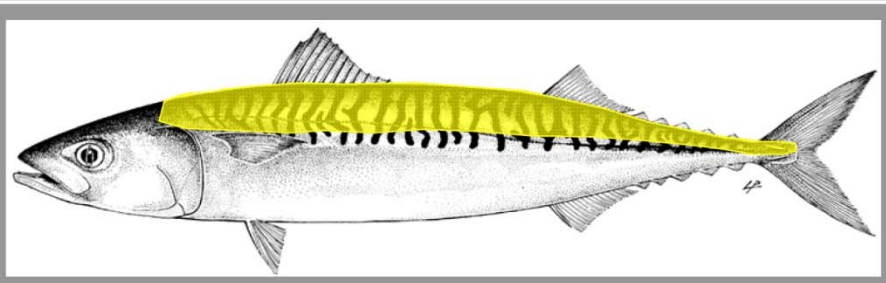
- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** Los parásitos están bastante localizados en la musculatura ventral, aunque se ha observado algún parásito aislado en la musculatura dorsal.

- **Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Trematodiasis



LOTE:

- Modo de presentación: fresco/entero.

- Procedencia: FAO 27 (ICES), 43°58'N 07°12'W.

PARÁSITO:

- **Agente etiológico:** *Didymozoidae.*

- **Información de interés:** Se vieron casos aislados de este parasito, pero siempre en el rango medio de talla de los peces estudiados.

- **Prevalencia:** accidental (<10%).

- **Tipo de afección:** calidad comercial.

MEDIDAS DE GESTIÓN:

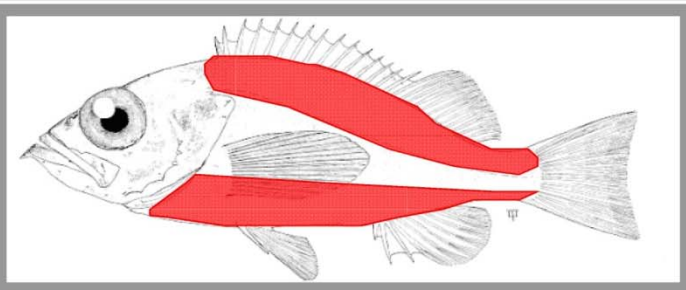
- **Puntos de Control Crítico:** se distribuyen por la musculatura dorsal, y especialmente en las regiones torácica y lumbar.

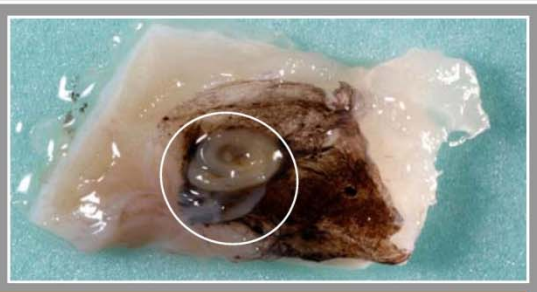
- **Medidas Correctoras:** al considerarse como casos aislados, se recomienda ampliar el muestreo y potenciar la profilaxis (adecuada congelación y/o cocinado) antes de emprender medidas correctoras.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

Sebastes mentella

Nematodiasis





LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. XIVb, 62°20'N 30°35'W.

PARÁSITO:

- Agente etiológico: *Anisakis simplex.*

- **Información de interés:** a medida que aumenta el calibre, aumenta el número de parásitos.

- **Prevalencia:** baja (10-20%).

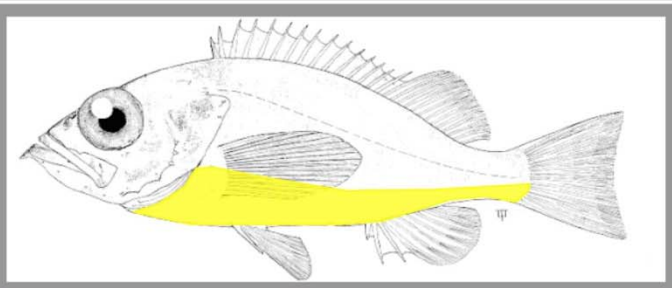
- **Tipo de afección:** calidad higiénico-sanitaria.

MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** localizado tanto en musculatura dorsal como ventral; especialmente en cavidad y musculatura perivisceral.

- **Medidas Correctoras:** Commission Regulation (EC) No 2074/2005 (EC 853/2004 rev.) ordena el examen visual y recomienda la eliminación de los parásitos visibles y de todo aquel pescado manifiestamente contaminado.

Copepodiasis



LOTE:

- Modo de presentación: congelado/entero.

- Procedencia: FAO 27 (ICES), Sub. XIVb, 62°20'N 30°35'W.

PARÁSITO:

- Agente etiológico: *Lernaeopodidae.*

- **Información de interés:** en la mayoría de los casos los parásitos se ven externamente.

- **Prevalencia:** accidental (<10%).

- **Tipo de afección:** calidad comercial.

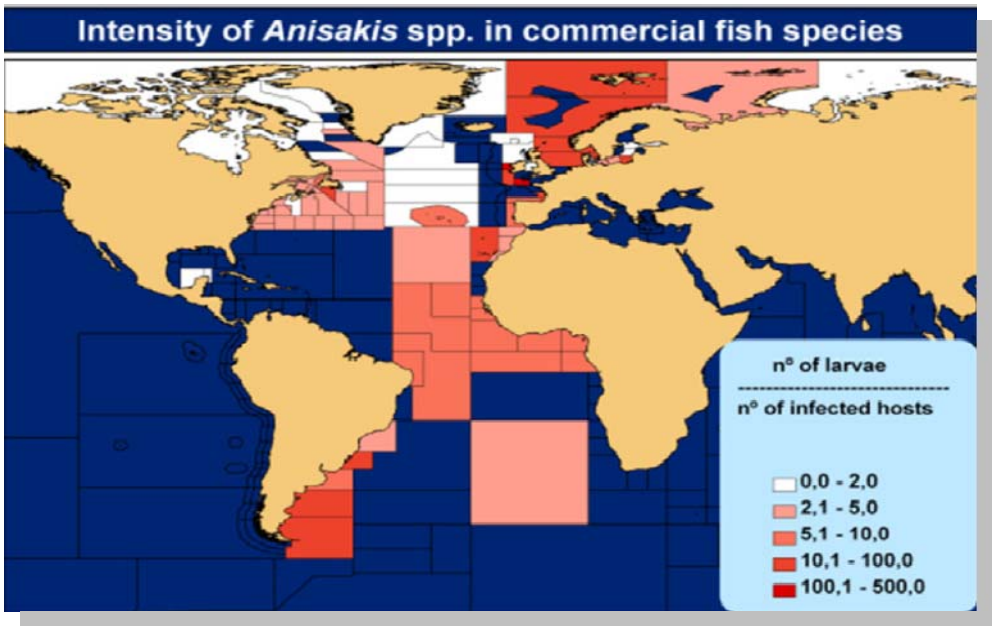
MEDIDAS DE GESTIÓN:

- **Puntos de Control Crítico:** normalmente localizado externa o internamente (en menos casos) en la inserción de las aletas pectorales, anales y adiposas.

- **Medidas Correctoras:** se aconseja extraer la musculatura de inserción de aletas pectorales, anales y adiposas cuando se observe externamente algún macroparásito o la alteración de la integridad de la epidermis en alguna de esas zonas.

Fuente: EPISTOCK. Proyecto INCITE-44.02.741A.771.0 de la Xunta de Galicia, coordinado por CETMAR (Centro Tecnológico del Mar)

All the sheets, which will shortly be incorporated into the website as descriptive fish species evaluating reports, constitute the starting point for the elaboration of similar sheets from any kind of potentially parasitized fish sample/lot that requires to be evaluated by the PARCODE's service "Advice". Moreover, this kind of sheets constitutes in itself the basis for the creation of dynamic risk maps which are considered a valuable consulting tool for the fishing sector.



See a more detailed example of these maps in Figure 2.2 (Chapter 2)

"Services":

"**Advice**" provides the user a continuous flow of intelligent and updated information directly from translational research studies on fish stocks with geo-referenced character. This service supplies inspection, analysis and identification of fish parasites to incorporate these skills into continuous improvement programs of enterprises, as well as education, training and self-control of workers of the sector. The two specific services that "Advice" offers are:

(a) "Diagnóstico molecular geo-referenciado y data minning" ("Georeferenced parasitic diagnosis of specific lots of fish and data-mining"):

This service provides comprehensive, personalized and confidential forward-looking information from lots or individual fishing products. To ensure traceability, this service offers risk analysis in origin. This tool allows generating dynamic risk maps from epidemiological databases.

To request the service it is only necessary to follow 8 steps:

1-2. "Rellenar" ("Fill in") and "Enviar" ("Send"):

To request the service of Inspection and Molecular Diagnosis to be carried out in the laboratory of ECOBIOMAR (Institute of Marine Research - CSIC) in Vigo, it is necessary to fill in the form available in the website, print it and attach it to ship the product to analyze to the postal address provided.

3. “Inspeccionar” (“Inspection”), “Analizar” (Analysis”) and “Identificar” (“Identification”):

Once the sample has been received in the laboratory, we proceed to the inspection, macroscopic and microscopic examination of the parasites present, molecular analysis, and identification by phylogenetic study of the sequences obtained in each case.

4-7. “Contactar” (“Contacting”), “Informar” (“Reporting”), “Asesorar” (“Advising”), “Mostrar” (“Showing”) and “Ayudar” (“Helping”):

The laboratory prepares a report which is transferred to the applicant company. A complete dossier including information, photos related to parasites or parasitic diseases object of the study, and advice giving the best solution to the problem, are also provided.

(b) “Paquete de mejora continua de la calidad entendida como inocuidad no percibida” (“Advice on continuous improvement of the quality, defined as no perceived food safety”).

It integrates comprehensive assistance for ongoing monitoring to fishing companies. The request for such service involves the implementation of a surveillance, warning and long-term analysis program (VAP) for parasitized fishery products defined by users. Methodologies for detection, mitigation and elimination of these parasites are also included for one year.

This package includes 3 services:

1- On-site visit: study of the problem in the processing facility.

2- Fish lots inspection, georeferenced parasitic diagnosis and data-mining.

3- Supply of suitable technical solutions to each case.

By completing the following application form, interested associated users (individuals or companies) may ask for information about this service or contact to demand it directly. Each request is evaluated and a no obligation quote is provided in every case.

"**Updating**" provides all users updated information on the following aspects:

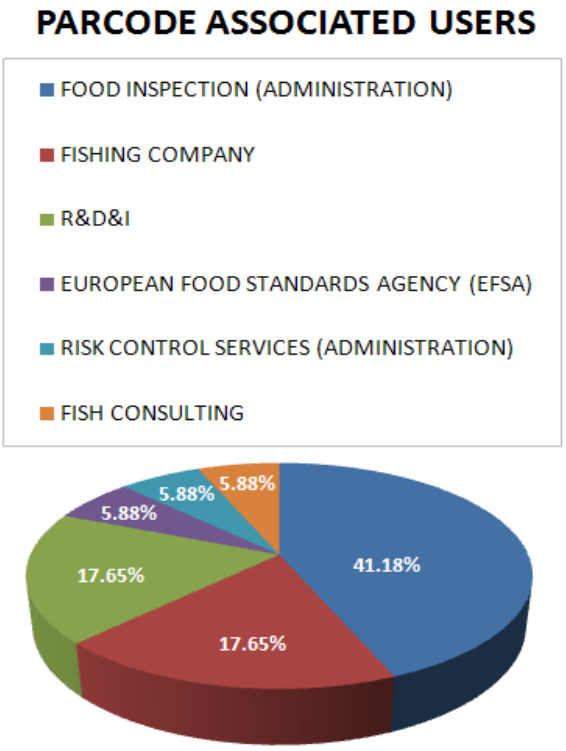
- Grants, scholarships and fundings in which actions of R&D&I related to parasites in fishery products arise.
- R&D&I activities and programmes.
- News and events from the fishing sector.
- Online discussion forum. Promoting a laboratory of ideas that should be as a knowledge broker, and a facilitator of the debate, by providing information and capacity-building.
- Face-to-face meeting and discussions. These events will have the aim of enhancing professional knowledge and synergies between companies, research field and the competent authority. It will help to define meeting channels to resolve conflicts and to establish strategic lines of action, becoming a standard-setter to forge common agreements on emerging parasites management.
- Translational research workshops. These seminars are presented as proof of concept and beta-testing by running fast counseling procedures (Rapid Assessment Survey methodology). These kinds of infodays promote collaboration among sector companies and research centers, allowing an industrial scaling of scientific knowledge that ultimately result in an innovation for the company.

Concerning the service "**Visualization**", the admission through a pin code, allows associated users, the access and visualization online of scientific and technological audiovisual material related to the

management of parasites in fishery products. This type of divulgative documentary material is continuously being updated.

"**Standardisation**" (under construction), has the aim of offering advice on rules and standards applicable for parasites in fishing companies. PARCODE managers are working with Bureau Veritas Spain (Division of Fisheries and Aquaculture) in the drafting and development of the documentary material needed for this purpose.

Furthermore, numerous professional users have submitted a request for admission into the platform as associated users or partners. Most of them requested the access during 2012 when the symposium, R&D&I forum and the round tables were held. The following graph illustrates the percentage of users associated according to the professional activities performed. No consumer has expected to associate.



10.4. DISCUSSION

Scientific excellence requires investment in R&D to improve and expand the scientific knowledge base, with regard to acquire a sound scientific basis for policy and regulation on food safety. The EU's Food Safety policy states in Chapter 3 of the White Paper of Food Safety (1999) its intention to carry out improvements in the areas of the rapid alert system, food safety research and the provision of scientific advice, among others. Moreover, the creation of a network of national scientific agencies and institutions in Member States in charge of food safety, with the Authority at its centre, and build upon their expertise, is designed to ensure the best and most effective use of existing structures and resources (Point 51 of Regulation (EC) 178/2002 and Point 54 in Chapter 4 of the White Paper of Food Safety, 1999).

PARCODE platform is a proof of concept of a practical implementation in the fisheries sector at the Galician level, and that is the main reason why it currently is only available for the Spanish version. At the same time, the accessible local infrastructure, personnel, laboratory and technical capacity for carrying out an elevated quantity of services, are limited. Furthermore, the fact that numerous requests to join the platform took place during 2012, just after the Symposium, R&D&I Forum and the round tables, means that more meetings must be organized in order to advertise and dynamize the network. No consumer has requested to associate because no information has been publicized at such level of user.

Food control authorities need to better value the role of science in the risk-based approach, and to take advantage of scientific resources in the international community (FAO, 2003). In fact, as Art. 11 of Regulation (EC) 853/2004 states, scientific evidences recommending health standards or checks to protect public health should be layed down whenever possible. As an example of this, Annex III concerning fishery products may be amended or supplemented to take account scientific and technical progress (Art. 17 of Regulation (EC) 854/2004). Moreover, as Commission Regulation (EU) 1276/2011 reports, when there is availability of new scientific evidences and practical experience, it is appropriate to amend legislative requirements in order to take them in account, as previously done on Regulation (EC) 853/2004 in relation to EFSA Opinion (2010).

PARCODE platform has been intended and created with the purpose of contributing to convert scientific findings and technological advances into industrial and commercial success, by encouraging users to use the information contained and to request the proposed services for their own interests and advice in terms of food safety. Along the same lines, the website here presented has been outlined and succeeded in becoming an easy to understand, user-friendly, dynamic, relevant and valuable tool, as many of the users have already described. If over time the platform becomes more relevant, users demand a higher quantity of services, and technical conditions improve, PARCODE website would immediately be translated to more languages and would be implemented and adapted to a wider user country range.

10.5. REFERENCES

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CHAPTER 11

Conclusions

The Horizon scanning work revealed that the *status quo* to manage fish parasites in the production-to-consumption food pathway is unsatisfactory and there is a lack of consensus and standardization for parasite inspection at fishing industries. A need for a tough and progressive program of unified standards concerning self-control more closely monitored by the authorities, has been increasing since food safety co-responsibility was transferred from governments to companies. The potential integration of parasitic epidemiological information and new methodologies of categorization included in HACCP plans opens the possibility to renew knowledge, predicting and preventing fish rejections and zoonoses, and contribute to enhance public consciousness and the success of control measures.

Specific conclusions are:

Epidemiology

A "dirty list" of zoonotic and/or economically-important parasite species is infecting major wild fish stocks of commercial interest in NE Atlantic fishing areas. Among them:

- The nematode of the genus *Anisakis,* well-known for its public health and economic concern, has been detected in 58-87% of fish species analysed. *Merluccius merluccius* and *Micromessistius poutassou* are the stocks with the highest prevalence and the greatest seasonal variability in mean abundance and density. Parasitic variations between fishing grounds, seasons or species must be considered for the creation of risk maps and related diagnostic tools within HACCP programs.

- Microsporidian xenomas found in *Lophius piscatorius* and *Lophius budegassa* were identified as *Spraguea* sp. in all cases. The commercial impact of *Spraguea* xenomas on fish quality represents a relevant industrial concern due to European regulations, but mostly by reason of unpleasant appearance of infected fishes.

- *Pennella instructa* has been genetically and morphologically diagnosed as the only copepod species parasitizing the Portuguese and Spanish stocks of Atlantic swordfish, *Xiphias gladius,* inspected. Since it is one of the most important parasites in terms of fish commercial quality, proactive measures and more effective corrective solutions are urgent needs to mitigate the high economic losses it causes.

Technology for parasite detection

- The absence of statistical significant relationship between the number of gut and muscular parasites, demonstrates the low accuracy of visual inspection as the commonly recommended method required by EU legislation to detect nematode larvae in the flesh of fish. Furthermore, the lack of effectiveness of the washing practice of gut, in terms of *Anisakis* spp. removal, creates difficulties regarding law enforcement.

- The new artificial digestion method based on liquid pepsin format, is a faster, cheaper, handier, more sensitive, efficient and accurate tool than the widely used procedure recommended by the Codex Alimentarius, and must be taken into account for the improvement of current screening programs for fish parasite prevention.

- Confocal studies, through the description of the fluorescent emission pattern and basic principles of auto-fluorescence of anisakids larvae, have provided substantial improvements to the UV-light examination procedure concerning visibility and resolution of nematode parasites. The reliability, effectiveness, work speed, user-friendliness, low investment cost, and diagnostic specificity, allow considering this enhanced detection method as a further step towards the development of a definitive and adapted industrial tool to be implemented in self-control programs at the fishing industry.

- Liquid nitrogen, formalin and particularly microwave have demonstrated to be effective treatments to be applied to nematode parasites before UV-light inspection, due to their efficiency in breaking parasitic cuticle and allowing the visibility of lipofucsin granules (fluorescence) under an UV-light source.

Risk Analysis

- A novel added-value and versatile technological tool (SADE) has been successfully designed as a proof-of-concept to be fitted and adapted to any commercial fish lot, and used to propose new prevention measures and risk mitigation strategies. This simple scoring-based system categorizes the parasitic infestation in fish individuals and stocks, for its use within HACCP programs.

Data management

- "PARCODE" is a thematic platform in website format, created for transferring and disseminate knowledge among seafood industry, food authorities (fish inspectors), consumers and researchers. It has succeeded in becoming an easy to understand, user-friendly, dynamic, relevant and valuable informative tool which contributes, among other tasks, to convert scientific findings and technological advances into industrial and commercial achievement. Collaborative translational research, professional training and a stable network performance based on shared software to provide multi-level information, are key drivers to stimulate technological transfer and innovations, in order to help minimizing the risk of parasites presence in fish products with public health and economic concern.